Metabolism and regulation of tetrahydrobiopterin and its implications for BH4-responsive hyperphenylalaninemia and BH4-deficiencies

Abstract

The autosomal recessive inherited, metabolic disorder phenylketonuria (PKU) is caused by a deficiency of the enzyme phenylalanine hydroxylase (PAH) - a key enzyme in the catabolism of phenylalanine. PKU patients present with noxiously increased concentrations of the amino acid phenylalanine in the plasma, leading to a so-called hyperphenylalaninemia. This disorder is treatable by avoiding uptake of phenylalanine. As this amino acid is naturally ubiquitously present in normal nutrition, patients have to follow a very strict and artificial diet in order to evade the grave consequences, such as severe mental retardation, they would have to expect otherwise. In the 1970s it was realised that there exists another cause of hyperphenylalaninemia which led to the detection of tetrahydrobiopterin deficiency. The connecting link between these two disor-ders is tetrahydrobiopterin (BH4), the natural cofactor of phenylalanine hydroxylase and other members of the aromatic amino acid hydroxylases, enzymes pivotal for catecholamine and serotonin biosynthesis. BH4 serves also as an essential cofactor for other enzymes (nitric oxide synthase and glyceryl-ether monooxygenase) and has additional functions on a cellular level. In recent years an other new variant of hyperphenylalaninemia/PKU was described (BH4-responsive HPA/PKU). Patients with this type of PKU are characterised by a marked reduction and normalisation of the increased phenylalanine concentration after oral loading with BH4. This finding opened a new perspective for a pharmacological treatment of so-called BH4-responsive HPA/PKU, as an alternative to the phenylalanine restricted diet which has notoriously a low compliance. With BH4 tested in medication of BH4-responsive HPA/PKU patients and being already used to treat BH4-deficient patients, our interest in the effects of exogenous BH4 on the organism was aroused. We started a project to study the influence of BH4, its metabolism and regulation. In the course of our study, we developed a new method for the measurement of different pterins (neopterin, biopterin, and pterin) in dried blood spots, which could be of use as an alternative in the screening for BH4 deficiencies. We identified new patients with GTP cyclohydrolase I deficiency, 6-pyruvoyl-tetrahydropterin synthase deficiency, and dihydropteridine reductase deficiency using this method. Extensive pharmacokinetic studies of BH4 have been performed in animal models but only few parameters are known from studies in humans. With the dried blood spots method we analysed the pharmacokinetics of orally administered BH4 in 71 patients with hyperphenylalaninemia and calculated a rapid absorption- (1.1 h) and distribution phase (2.5 h) and a slower elimination phase (46 h). Previous findings of others, that BH4-responsiveness was higher among patients with mild PAH mutations, could be confirmed.In a third part we looked at the molecular genetics of BH4 responsive HPA/PKU patients. By virtue of data mining in the BIOPKU database we identified 60 different mutations associated with BH4 responsiveness and analysed the frequency of potentially responsive genotypes and their dispersal in Europe. We estimated an average of 55% responsive amongst HPA/PKU patients in Europe. We also studied the outcome and made a long-term follow-up of 36 patients with BH4 defi-ciency, 26 with a 6-pyruvoyl-tetrahydropterin deficiency and 10 with dihydropteridine reduc-tase deficiency, the two most common forms of BH4-deficiency. Our data suggested that diagnosis within the first month of life is essential for a good outcome and that low 5-hydroxyindolacetic acid and homovanillic acid values in cerebrospinal fluid could be an indicator for the ongoing developmental impairment, even in absence of neurological symptoms. In a last part, we investigated in the effects of BH4 on the metabolism and regulation of en-zymes, either directly involved in the biosynthesis and regeneration of BH4 or otherwise associ-ated with BH4 metabolism. The experiments were performed employing various cell lines, which were treated by supplementation of BH4 and other agents with either stimulating or

inhibiting effects. In most investigated cell lines, after supplementation with cytokines, a significant and strong up-regulation (~50-fold) of the gene expression of GCH1 was found, the gene encoding for GTP cyclohydrolase I, the first and rate limiting enzyme in BH4 de novo biosynthesis. Furthermore, the expression of AKR1B1, involved in alternative pathway, was found to be upregulated (~4-fold). A slight but significant reduction of the transcription of QDPR, coding for the enzyme dihydropteridine reductase, was observed after supplementation with sepiapterin, known to be taken up quickly by cell cultures and intracellularly converted to BH4.

Metabolism and Regulation of Tetrahydrobiopterin and its Implications for BH_4-Responsive Hyperphenylalaninemia and BH_4-Deficiencies

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ABSTRACT

The autosomal recessive inherited, metabolic disorder phenylketonuria (PKU) is caused by a deficiency of the enzyme phenylalanine hydroxylase (PAH) – a key enzyme in the catabolism of phenylalanine. PKU patients present with noxiously increased concentrations of the amino acid phenylalanine in the plasma, leading to a so-called hyperphenylalaninemia. This disorder is treatable by avoiding uptake of phenylalanine. As this amino acid is naturally ubiquitously present in normal nutrition, patients have to follow a very strict and artificial diet in order to evade the grave consequences, such as severe mental retardation, they would have to expect otherwise.

In the 1970s it was realised that there exists another cause of hyperphenylalaninemia which led to the detection of tetrahydrobiopterin deficiency. The connecting link between these two disorders is tetrahydrobiopterin (BH_4), the natural cofactor of phenylalanine hydroxylase and other members of the aromatic amino acid hydroxylases, enzymes pivotal for catecholamine and serotonin biosynthesis. BH_4 serves also as an essential cofactor for other enzymes (nitric oxide synthase and glyceryl-ether monooxygenase) and has additional functions on a cellular level.

In recent years an other new variant of hyperphenylalaninemia/PKU was described (BH_4-responsive HPA/PKU). Patients with this type of PKU are characterised by a marked reduction and normalisation of the increased phenylalanine concentration after oral loading with BH_4. This finding opened a new perspective for a pharmacological treatment of so-called BH_4-responsive HPA/PKU, as an alternative to the phenylalanine restricted diet which has notoriously a low compliance.

With BH_4 tested in medication of BH_4-responsive HPA/PKU patients and being already used to treat BH_4-deficient patients, our interest in the effects of exogenous BH_4 on the organism was aroused. We started a project to study the influence of BH_4, its metabolism and regulation.

In the course of our study, we developed a new method for the measurement of different pterins (neopterin, biopterin, and pterin) in dried blood spots, which could be of use as an alternative in the screening for BH_4 deficiencies. We identified new patients with GTP cyclohydrolase I deficiency, 6-pyruvoyl-tetrahydropterin synthase deficiency, and dihydropteridine reductase deficiency using this method.

Extensive pharmacokinetic studies of BH_4 have been performed in animal models but only few parameters are known from studies in humans. With the dried blood spots method we analysed the pharmacokinetics of orally administered BH_4 in 71 patients with hyperphenylalaninemia and calculated a rapid absorption- (1.1 h) and distribution phase (2.5 h) and a slower elimination phase (46 h). Previous findings of others, that BH_4-responsiveness was higher among patients with mild PAH mutations, could be confirmed.In a third part we looked at the molecular genetics of BH_4 responsive HPA/PKU patients. By virtue of data mining in the BIOPKU database we identified 60 different mutations associated with BH_4 responsiveness and analysed the frequency of potentially responsive genotypes and their dispersal in Europe. We estimated an average of 55% responsive amongst HPA/PKU patients in Europe.

We also studied the outcome and made a long-term follow-up of 36 patients with BH_4 deficiency, 26 with a 6-pyruvoyl-tetrahydropterin deficiency and 10 with dihydropteridine reductase deficiency, the two most common forms of BH_4-deficiency. Our data suggested that diagnosis within the first month of life is essential for a good outcome and that low 5-hydroxyindolacetic acid and homovanillic acid values in cerebrospinal fluid could be an indicator for the ongoing developmental impairment, even in absence of neurological symptoms.

In a last part, we investigated in the effects of BH_4 on the metabolism and regulation of enzymes, either directly involved in the biosynthesis and regeneration of BH_4 or otherwise associated with BH_4 metabolism. The experiments were performed employing various cell lines, which were treated by supplementation of BH_4 and other agents with either stimulating or inhibiting effects. In most investigated cell lines, after supplementation with cytokines, a significant and strong up-regulation (~50-fold) of the gene expression of *GCH1* was found, the gene encoding for GTP cyclohydrolase I, the first and rate limiting enzyme in BH_4 *de novo* biosynthesis. Furthermore, the expression of *AKR1B1*, involved in alternative pathway, was found to be upregulated (~4-fold). A slight but significant reduction of the transcription of *QDPR*, coding for the enzyme dihydropteridine reductase, was observed after supplementation with sepiapterin, known to be taken up quickly by cell cultures and intracellularly converted to BH_4.

ZUSAMMENFASSUNG

Die autosomal-rezessiv vererbte Stoffwechselkrankheit Phenylketonurie (PKU) wird durch einen Defekt des Enzyms Phenylalaninhydroxylase (PAH) verursacht, das für den Katabolismus von Phenylalanin verantwortlich ist. Im Plasma von PKU Patienten wird eine gesundheitschädlich erhöhte Konzentration der Aminosäure Phenylalanin gefunden, was zu einer so genannten Hyperphenylalaninämie führt. Wird eine Aufnahme von Phenylalanin vermieden, lässt sich diese Krankheit gut kontrollieren, da diese Aminosäure aber omnipräsent in der normalen Nahrung vorliegt, müssen PKU Patienten eine spezielle, künstliche Diät halten. Wenn diese nicht strikt befolgt wird, drohen schwerwiegende Konsequenzen, wie Entwicklungsverzögerungen und geistige Behinderung.

In den 1970er wurde mit der Entdeckung der Tetrahydrobiopterin-Defizienz eine weitere Ursache für Hyperphenylalaninämien gefunden. Das verbindende Element dieser beiden Krankheiten ist das Tetrahydrobiopterin (BH_4), der natürliche Cofaktor der Phenylalaninhydroxylase und anderer Aromatischer-Aminosäure-Hydroxylasen, welche entscheidend an der Biosynthese von Katecholaminen und Serotonin beteiligt sind. BH_4 dient auch anderen Enzymen als essentieller Cofaktor (NO-Synthase, Glycerl-Ether Monooxygenase) und hat weitere Funktionen auf Stufe der Zelle.

In der jüngsten Vergangenheit wurde eine neue PKU Variante beschrieben (BH_4-sensitive HPA/PKU). Patienten mit dieser Form PKU bzw. HPA zeichnen sich durch eine deutliche Reduktion und Normalisierung der Phenylalaninkonzentration nach einer oralen Verabreichung von BH_4 aus. Diese Entdeckung eröffnete neue Perspektiven für eine medikamentöse Behandlung von PKU, als Alternative zur Diät mit reduziertem Phenylalaningehalt, die bekanntermassen eine schlechte Compliance aufweist.

BH_4 wird als Medikation von BH_4-sensitiven HPA/PKU Patienten getestet und wird in der Behandlung von Patienten mit BH_4-Mangel bereits seit einiger Zeit eingesetzt. Dies erweckte unser Interesse zu untersuchen, welche Einflüsse das exogen zugeführte BH_4 auf den Organismus hat und wir starteten ein Projekt, um die Effekte, den Metabolismus und die Regulation von BH_4 zu untersuchen.

Im Verlaufe unserer Studie entwickelten wir eine neue Methode zur Messung verschiedener Pterine (Neopterin, Biopterin und Pterin) in Trocken-Blut, die als alternative Methode im BH_4-

Defizienzen-Screening dienen könnte. Es war uns möglich, neue Patienten mit GTP Cyclohydrolase I Defizienz, 6-Pyruvoyltetrahydropterin Synthase Defizienz und Dihydropteridin Reduktase Defizienz mit dieser Methode zu identifizieren.

In früheren Studien wurden umfassende pharmakokinetische Untersuchungen mit BH_4 an Tiermodellen durchgeführt aber nur wenige Parameter waren von Studien mit Menschen bekannt. Mit oben erwähnter Methode analysierten wir die Pharmakokinetik von oral verabreichtem BH_4 in 71 Patienten mit Hyperphenylalaninämie und fanden eine schnelle Absorptions- (1.1 h) und Verteilungsphase (2.5 h), sowie eine etwas langsamere Ausscheidungsphase (46 h).

In einem dritten Teil untersuchten wir die molekulare Genetik von BH_4-sensitiven HPA/PKU Patienten. Mittels Data-Mining in der BIOPKU Datenbank identifizierten wir 60 verschiedene Mutationen, die wir mit einer BH_4-Sensitivität assoziieren konnten. Wir untersuchten die Genfrequenz von potentiell sensitiven Genotypen und deren Verteilung in Europa. Wir berechneten einen Anteil von 55% BH_4-sensitiven in der Gesamtheit der PKU Patienten in Europa.

Andere Studien fanden, dass besonders Patienten mit milden PAH Mutationen auf eine BH_4-Gabe ansprechen. Diese Forschungsergebnisse konnten wir bestätigen.

Weiter analysierten wir den Langzeit-Therapieerfolg von 36 Patienten mit einer BH_4-Defizienz, 26 davon litten an einem 6-Pyruvoyltetrahydropterin Synthase Mangel und 10 an einer Dihydropteridin Reduktase Defizienz, den zwei häufigsten Formen einer BH_4-Defizienz. Aufgrund unserer Daten kamen wir zur Feststellung, dass eine Diagnose im ersten Lebensmonat entscheidend für einen positiven Therapieverlauf ist und dass eine tiefe Konzentration von 5-Hydroxyindolessigsäure und Homovanillinsäure im Liquor cerebrospinalis, auch bei fehlenden neurologischen Symptomen als Indiz für eine anhaltende Beeinträchtigung der Entwicklung dienen könnte.

In einem letzten Teil haben wir die Effekte von BH_4 auf den Metabolismus und die Regulation von Enzymen studiert, die entweder direkt an der Biosynthese bzw. Regeneration von BH_4 beteiligt sind oder aber sonst mit der BH_4 Synthese assoziiert sind oder BH_4 als Cofaktor für ihre enzymatische Aktivität nutzen. Wir wollten wissen, ob durch BH_4-Gabe, wie das z.B. bei PKU Patienten der Fall ist, andere Stoffwechselsysteme beeinflusst werden. Zur Durchführung der Experimente machten wir von diversen Zelllinien Gebrauch, die wir durch Zugabe von BH_4

und anderer Reagenzien beeinflussten, was entweder zu stimulierenden oder inhibierenden Effekten des BH_4-Systems führte. In den meisten der untersuchen Zelllinien fanden wir, nach Zugabe von Zytokinen, eine starke, signifikante Erhöhung (~50-fach) der Expression von *GCH1*, dem Gen der GTP Cyclohydrolase I, die das erste und geschwindigkeitsbestimmenden Enzym der BH_4 *de novo* Biosynthese darstellt. Weiter wurde gefunden, dass die Expression des Gens *AKR1B1*, involviert im alternativen Stoffwechselweg von BH_4, ebenfalls induziert wurde. Nach Zugabe von Sepiapterin, das von Zellen rasch aufgenommen und in BH_4 umgewandelt wird, fanden wir tendenziell eine leichte Reduktion der Expression von *QDPR*, dem Gen, das die Dihydropteridin Reductase codiert.

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ABBREVIATIONS

5HIAA: 5-hydroxyindolacetic acid

5HTRP: 5-hydroxytryptophan

5MTHF: 5-methyltetrahydrofolic acid

BH_4: tetrahydrobiopterin

BIODEF: International Database of Tetrahydrobiopterin Deficiencies

BIOMDB: Database of Mutations Causing Tetrahydrobiopterin Deficiencies

BIOPKU: International Database of BH_4-responsive HPA/PKU

bp: base pair(s)

CSF: cerebrospinal fluid

CK: cytokines

DAHP: 2,4,-diamino-6-hydroxypyrimidine

DHPR: dihydropteridine reductase

DMEM: Dulbecco Modified Eagle Medium

EDTA: ethylendiamintetraacetat

GTPCH: GTP cyclohydrolase I

HPA: hyperphenylalaninemia

HVA: homovanillic acid

IFN-γ: interferon-γ

kb: kilobase pairs

kDa: kilo Dalton

L-dopa: levodopa (3,4-dihydroxy-L-phenylalanine)

NADH: nicotinamide adenine dinucleotide

NADPH: nicotinamide adenine dinucleotide phosphate

NO: nitric oxide

NOS: nitric oxide synthase

PAH: phenylalanine hydroxylase

PAHdb: PAH database

PBS: phosphate buffered saline

PBT: phosphate buffered saline + 0.1% Tween-20

PCD: pterin-4α-carbinolamine dehydratase

PCR: polymerase chain reaction

Phe: phenylalanine

PKC: protein kinase

PKU: phenylketonuria

PTPS: 6-pyruvoyl-tetrahydropterin synthase

qBH_2: quinoid dihydrobiopterin

SP: sepiapterin

SR: sepiapterin reductase

TH: tyrosine hydroxylase

TNF-α: tumour necrosis factor-α

TPH: tryptophan hydroxylase

Chapter **1**

INTRODUCTION

Phenylketonuria and hyperphenylalaninemia

Phenylketonuria: from discovery to therapy

Phenylketonuria (PKU) is a widespread autosomal recessive genetic disorder (OMIM 261600) which results from a deficiency of the hepatic enzyme phenylalanine-4-hydroxylase (PAH). It is characterised by mental retardation if untreated. Nowadays most developed countries routinely perform newborn screening to detect PKU (Scriver 2007).

The discovery of PKU was made in the year 1934 by the Norwegian physician Asbjørn Følling, when a mother of two mentally retarded children came to him for advice. Her seven-year-old daughter was only capable of speaking a few words, and had a purposeless way of moving around, the four-year-old son did not walk, was unable to fix his eyes on anything and his stool and urine habits were those of a baby. They both had a fair complexion and somewhat spastic extremities. A peculiar mousy odour was emanating from their body and urine. By classical, organic-chemical means, Følling proved that these two children and eight additional patients, suffering from mental retardation, excreted phenylpyruvic acid in their urine. Based on these observations he named the disease imbecillitas phenylpyrouvica (Følling 1994).

The following year this disease was renamed to phenylketonuria by S.L. Penrose (Hendriksz and Walter 2004; Penrose 1998) and in 1947 G.A. Jervis could prove that phenylketonuria is based on the deficiency to metabolise the amino acid phenylalanine to tyrosine (Jervis 1953). Some years later Woolf and Vulliamy (Woolf and Vulliamy 1951) suggested that a phenylalanine-restricted diet might be beneficial in preventing patients from neurological damage. This hypothesis was first successfully tested in 1953 by German paediatrician H. Bickel, who treated effectively a patient with phenylalanine low diet (Bickel 1996; Woolf et al.

1955). These two characteristics made phenylketonuria a paradigm for treatable genetic diseases (Scriver and Waters 1999).

Hyperphenylalaninemia

In the 1960s the introduction of newborn screening for PKU started, using then a simple bacterial growth test with *bacillus subtilis*, not until then it was considered that classification in PKU might become necessary one day (Guthrie and Susi 1963). Ever since, PKU has proven to be a disorder with a broad spectrum of different phenotypes (Marsden and Levy 2006). The most severe form, the classical PKU, shares with the other, less grave forms, the fact that blood phenylalanine levels are increased. This condition is called hyperphenylalaninemia (HPA); today serum concentrations above 120 µM are regarded as pathologically increased. In the following years, it was found that HPA is genetically a heterogeneous disorder; 98% of the patients suffering from HPA present with a defect of hepatic enzyme PAH. To date (2007) over 500 different mutations in the encoding gene *PAH* have been detected and recorded in the locus-specific PAHdb database (http://www.pahdb.mcgill.ca/). Most patient are compound heterozygous, and the vast number of possible combinations, of alleles with grave or less serious mutations, partially explains the variety in degree of severity of this disorder.

In the 1970s it became recognised that in the remaining 2%, HPA was caused by a lack of sufficient tetrahydrobiopterin (BH_4), the natural cofactor of the enzyme PAH (Kaufman et al. 1975, 1978; Tada et al. 1970).

The classification of the grade of HPA is mainly based on the plasma concentrations of phenylalanine in the untreated patient. There is no world wide consensus on cut-off values, but generally following classification, in descending order in respect of phenylalanine (Phe) concentration, applies: 1) classical PKU (Phe >1200 µM), 2) moderate/mild PKU (Phe 600-1200 µM), and 3) non-PKU/mild HPA (Phe <600 µM) (Table 1). Besides of these, there are also the BH_4 deficiency and transient forms of HPA due to: prematurity, iatrogenesis, neonatal liver disease, and maternal hyperphenylalaninemia (Marsden and Levy 2006).

HPA as a metabolic consequence of PAH deficiency can have a toxic effect on the brain due to a drastic increase of plasma phenylalanine, which may even exceed concentrations of 1200 µM, but there is evidence that levels below 600 µM in untreated patients may not be harmful for the cognitive development (Scriver and Kaufman 2001; Scriver et al. 1994). Patients with a mild

HPA do not need to follow a phenylalanine restricted diet; nevertheless a thoughtful approach to intake of protein-rich nutrition is recommended (Matalon and Michals-Matalon 2006).

PKU/HPA is, as mentioned before, an autosomal recessive disorder, inherited in a Mendelian fashion, with an average incidence of about 1:10'000 newborns in Europe. The occurrence can vary considerably between different countries, for example on Ireland an incidence of approx. 1:4500 was stated, whereas in Finland the ratio was estimated to be below 1:100'000 (Guldberg et al. 1995; Zschocke 2003).

The name phenylketonuria was coined in recognition of the unusual metabolic by-products resulting from the inability of HPA patients to metabolise dietary phenylalanine to tyrosine. Instead phenylalanine is accumulated in the blood and partly degraded to phenylpyruvate, phenylacetate and phenyllactate, which all can be found in the patient's urine. In addition to the toxic effects of phenylalanine, it can outcompete other large neutral amino acids (such as tyrosine, tryptophan and branched chain amino acids etc.) in the transport through the blood-brain barrier into the brain, as the responsible carrier protein has the lowest K_m for phenylalanine. This leads to an increased influx of phenylalanine into the brain and to a reduction of the already decreased concentrations of tyrosine. It was suggested that these two factors cause brain protein synthesis to decrease, and stimulate myelin turnover on the other hand, besides of the occurrence of further abnormalities in the amine neurotransmitter systems, explaining brain damages seen in untreated patients (Matsuo and Hommes 1987; Surtees and Blau 2000).

Phenylalanine-4-hydroxylase

The cause of the disorder discovered by Følling, is a deficiency of the enzyme phenylalanine-4-hydroxylase (PAH; EC 1.14.16.1). It is encoded by the gene *PAH* on chromosome 12q22-q24.2. It contains 13 exons, spanning about 171 kb, which translate into a 52 kDa protein with a primary sequence of 452 amino acids (Scriver 2007).

PAH catalyses the para-hydroxylation of L-phenylalanine to L-tyrosine using BH_4 as a cofactor, and O_2 as additional substrate. In addition to this, PAH shows a strict requirement for a non-heme iron. Under physiological conditions, PAH consumes about 75% of the phenylalanine taken up from food or gained through protein catabolism (Scriver and Kaufman 2001). It is functionally active as a homotetramer, and in solution it is present in equilibrium with dimers

(Kaufman 1993; Pey and Martínez 2006). Its monomeric subunit (52 kDa) displays three different domains: the regulatory domain, located at the N-terminus, providing a serine residue (Ser16) as a phosphorylation site, and responsible for positive cooperativity induced by phenylalanine, respectively for an negative regulatory effect by BH_4; second, the catalytic domain, containing the active site with a coordinated iron atom, and the binding sites for phenylalanine and BH_4; third, the tetramerization domain, facilitating oligomerisation via interaction of coiled-coil motifs (Pey and Martínez 2006).

PAH together with tyrosine-3-hydroxylase (TH; EC 1.14.16.3), and the two isoforms of tryptophan-5-hydroxylase (TPH1, peripheral; TPH2, neuronal; EC 1.14.16.4) belongs to the family of the aromatic amino acid hydroxylases (Fitzpatrick 2000; Walther and Bader 2003).

The major pool of PAH activity in mammals is the liver, controlling the overall phenylalanine homeostasis. In humans, also other organs have been reported to show PAH activity or at least to express *PAH* mRNA, as there are the kidney, brain, pancreas and melanocytes (Pey and Martínez 2006).

It has been proposed that there would be enough PAH activity present in the liver to deplete plasma phenylalanine levels within several minutes if the enzyme was constantly and fully active. In order to maintain the phenylalanine homeostasis *in vivo*, the activity of PAH is tightly regulated by several mechanisms. Aforementioned positive cooperativity of PAH and phenylalanine (Hill coefficient, $h \approx 2$) is thought to be of major physiological relevance in the control mechanism of phenylalanine homeostasis in the blood (Kappock and Caradonna 1996; Kaufman 1993). Upon binding of phenylalanine in concert with phosphorylation, the enzyme undergoes conformational changes, which moves the autoregulatory sequence away from the active site, and becomes thereby activated (Kappock and Caradonna 1996; Kobe et al. 1999; Miranda et al. 2004; Thórólfsson et al. 2003). On the other hand BH_4 acts as an allosteric inhibitor, keeping the enzyme in a low-activity state. This PAH·BH_4 complex is thought to keep the enzyme in a latent form, to allow for a rapid activation in response to increased intra- and extracellular (plasma) phenylalanine concentrations. Binding of BH_4 leads to a conformational change, which triggers a closing of the entrance to the active site by the N-terminal regulatory domain (Mitnaul and Shiman 1995; Pey and Martínez 2006; Pey et al. 2004). The third major contributor to PAH regulation is phosphorylation at Ser16 in the N-terminal regulatory domain. It was found to be mediated by cAMP and Ca^{2+}/calmodulin dependent protein kinase and leads to an increased basal activity and apparent affinity for the substrate (Kaufman 1993; Miranda et al. 2002). As showed *in vitro*, the rate of the phosphorylation is increased in the presence of phenylalanine and decreased by BH_4

Phenylalanine restricted diet

The genetic disorder PKU or HPA is treatable with a special diet, low in phenylalanine content. Even patients with severe PKU can develop normal cognitive abilities, when the diet treatment is started at early infancy and the blood phenylalanine concentration in the blood is controlled and maintained on a close to normal level (Waisbren et al. 2007).

Nevertheless there are several drawbacks to the phenylalanine restricted diet: To prevent progressive loss of intellectual functions it has to be followed for a lifetime (Smith et al. 1978). Adding to this, it is drastically interfering with a normal lifestyle and demands a high level of discipline, as patients are not allowed to eat meat, fish, eggs, dairy products, pasta, and bakery products. Sweets and vegetables are permitted to a certain degree. The diet consists of a phenylalanine free protein substitution, minerals, vitamins, trace elements, protein reduced special products and other nourishments with low protein content, and is mostly regarded as not being savoury at all (MacDonald et al. 1997; Harding 2000; 2001). In the light of these obstacles, it is no surprise that compliance is in general rather low, and patients tend to be more prone for psychological problems like depressions (Smith and Knowles 2000; Walter et al. 2002; Weglage et al. 1996).

Maternal PKU

An international study in the 1980s (Lenke and Levy 1980) showed a high frequency of mental retardation (92%), microcephaly (73%) low birth weight (40%), and congenital heart disease (12%) in offspring of mothers with PKU, as they suffered, when still being a foetus, from the high concentrations of phenylalanine from the maternal metabolism. Therefore, treatment strategies had to be developed for pregnant women with PKU. For them dietary restrictions had become an absolute requirement during pregnancy to prevent the occurrence of this so-called maternal PKU, leading to developmental abnormality and mental impairment of the unborn due to increased phenylalanine levels in the foetus' body (Koch et al. 2003; Platt et al. 2000; Sheard 2000).

Alternative treatment

Several strategies have been developed as alternatives to treatment with phenylalanine restricted diet. One of the most promising ways is somatic gene therapy. Recent publications reported for the first time successful application of adeno-associated virus vectors, targeted to the liver, leading to complete correction of hyperphenylalaninemia in a PKU mouse model. The viral vectors were used to deliver *PAH* cDNA into the liver cells, leading to a long-lasting expression of PAH activity in these otherwise PAH-deficient mice. Different similar strategies have been employed (Chen and Woo 2007; Ding et al. 2004, 2006; Harding et al. 2006; Oh et al. 2004). To eliminate the potential risk of side-effects due to the nature of the vector, other techniques to deliver the gene construct are now object of ongoing research.

A totally different approach to reduce phenylalanine in patients is the so-called enzyme substitution therapy. Sarkissian and others (Sarkissian and Gámez 2005; Sarkissian et al. 1999) and similarly Liu and others (Liu et al. 2002) investigated in the feasibility of replacing PAH by the non-mammalian, recombinant phenylalanine ammonia lyase (PAL). This enzyme converts phenylalanine to the harmless metabolites, trans-cinnamic acid and trace ammonia. Taken orally and when non-absorbable and protected, PAL lowered plasma phenylalanine in a mutant hyperphenylalaninemic mouse model. Subcutaneous administration of PAL was also tested with an even more substantial lowering of plasma levels and significant reduction in brain phenylalanine levels; however the metabolic effect was not sustained following repeated injections due to an immune response.

A third alternative or supplementation to the low phenylalanine diet to treat PKU patients, is the oral administration of large neutral amino acids (LNAA, i.e. valine, isoleucine, leucine) competing with phenylalanine for the transport across the blood-brain barrier, thereby preventing excessive influx of phenylalanine to the brain. The benefit applying LNAA is still a matter of controversial debate but they may be of some use for patients not complying with the diet treatment (Matalon et al. 2003; Schindeler et al. 2007).

The discovery of a new variant of PKU opened up new vistas for pharmacological treatment of PKU with BH_4, the natural cofactor of the defective enzyme PAH. See next paragraph.

Tetrahydrobiopterin responsive hyperphenylalaninemia

The disorder PKU manifests itself in a broad spectrum of phenotypes, from severe classical PKU to less malignant forms, such as mild hyperphenylalaninemia. In the year 1999 a new variant named BH_4 responsive HPA/PKU, was described for the first time by Kure (Kure et al. 1999). He described four patients with hyperphenylalaninemia who responded to oral administration of BH_4 with a reduction of their plasma levels of phenylalanine. Soon thereafter other studies suggested that BH_4 can be successfully used in the long-term treatment of HPA patients as an alternative to phenylalanine restricted diet (Bélanger-Quintana et al. 2005; Cerone et al. 2004; Hennermann et al. 2005; Lambruschini et al. 2005; Muntau et al. 2002; Shintaku et al. 2004; Steinfeld et al. 2004; Trefz et al. 2001; 2005)

Patients are defined as BH_4-responsive if they reduce their phenylalanine plasma levels after a BH_4 loading test. There is no strict norm determining cut-off levels of responsiveness or the exact amount of BH_4 used for the test. It seems generally acceptable to use a 30%-reduction within 24 hours as cut-off, after oral administration of 20 mg/kg BH_4. As shown in (Fiege and Blau 2007) also within shorter timeframes or higher cut-off values, responsible patients can be identified. Furthermore this study suggested that BH_4 responsive patients are mostly found amongst mild HPA to mild PKU patients. Nevertheless also several patients with classical PKU responded to BH_4 loading.

Apparently in these patients, the super-physiological concentration of BH_4 activates the deficient enzyme PAH (Pey and Martínez 2005). There are several hypotheses concerning the exact mechanism of BH_4 responsiveness. For instance, high dose of BH_4 may compensate for decreased affinity of the deficient PAH. BH_4-responsive patients display a high degree of heterogeneity at the genetic level but only in some cases so-called K_m mutants could be identified. A recent study (Pey et al. 2007) attributes the main molecular mechanism underlying BH_4 responsiveness to a chaperone-like effect, by which BH_4 increases the stability of mutant PAH proteins (thermal stability and protection against proteolytic degradation and oxidative inactivation). Considerable residual activity is further proposed as being an important characteristic. On the whole BH_4 responsiveness appears to be multifactorial (Aguado et al. 2006; Blau and Erlandsen 2004; Erlandsen and Stevens 2001; Erlandsen et al. 2003; Kure et al. 2004; Pey et al. 2004; Steinfeld et al. 2003; Thöny et al. 2004).

Since the first report on patients with BH_4-responsive HPA/PKU, it hast been shown that a considerable percentage of patients with HPA respond to pharmacological doses of BH_4

(Bernegger and Blau 2002). Mainly patients with mild clinical phenotypes benefit most from treatment with the cofactor, which allows more than 80% of them to discontinue their phenylalanine low diet. But there is also evidence that some patients suffering from severe forms of PKU respond to BH_4. For this group of patients – who often show a partial response to pharmacological therapy – a combined treatment of a less stringent diet together with administration of BH_4, might be valuable (Muntau and Gersting 2006).

Tetrahydrobiopterin deficiencies

With the detection in the 1970s of several patient apparently suffering from PKU, but who did not respond to diet treatment, it was speculated that these formally called untypical PKU patients might be affected by a new form of HPA caused by a deficiency of the BH_4 metabolism. Features they had in common were that they, regardless of an early diagnosis for HPA, did not react with a reduction of the phenylalanine levels upon treatment with phenylalanine restricted nutrition. They developed progressive neurological symptoms and many died at early age (Bartholomé 1974; Bartholomé et al. 1977; Brewster et al. 1979; Danks et al. 1979; Kaufman et al. 1975, 1978; Smith and Lloyd 1974; Smith et al. 1975; Tada et al. 1970).

Nowadays several variants of these BH_4 deficiencies are known and characterised, and have been catalogued in the databases BIODEF and BIOMDB, respectively (www.bh4.org). The first one lists worldwide data from screening over the last 30 years. It includes information on patient's age, ethnic origin, information about the parents, siblings, laboratory values, treatment, clinical symptoms, and so on. This database is linked with the second one, the BIOMDB, where all the gene mutations of the corresponding patients are mapped. More than 500 patients have been diagnosed as a result of selective screening during the last 30 years. 58% of them presented with PTPS deficiency, 30% with DHPR deficiency, and each about 4% suffered from PCD- or GTPCH-deficiency (for definition of the different deficiencies see below) (Blau and Dhondt 2006).

Nomenclature of tetrahydrobiopterin deficiencies

BH_4 deficiencies, previously termed "atypical PKU", form a very heterogeneous group of different disorders (Table 1). They present with diverse clinical and biochemical characteristics

(Blau et al. 2001). The affected enzyme in the first place, the type of the mutation, its severity, the outcome of a BH_4 challenge and the response to therapy are all criteria to be considered when defining as a specific variant. Accordingly to the actual need for treatment with neurotransmitter precursors, the terms severe, mild or peripheral should be used to describe the severity (Blau 2006).

Two principle distinctions can be made: BH_4 deficiencies with HPA and BH_4 deficiencies without HPA. The first can all be detected by neonatal screening for PKU or HPA. Selective screening for BH_4-deficiency is of great importance in every newborn with plasma phenylalanine levels higher than 120 μM and in older children showing neurological symptoms of unclear origin (Blau et al. 1996; Zurflüh et al. 2005).

Tetrahydrobiopterin (BH_4) deficiencies with hyperphenylalaninemia (HPA)

Four autosomal-recessively inherited enzyme defects belong to the group of BH_4 deficiencies with HPA: GTP cyclohydrolase I (arGTPCH) deficiency, 6-pyruvoyl-tetrahydropterin synthase (PTPS) deficiency, carbinolamine-4α-dehydratase (PCD) deficiency and dihydropteridine reductase (DHPR) deficiency. They can be further divided into two groups, mild or severe, depending on the presence or absence of normal CNS monoamine neurotransmitter metabolism. In the latter group, patient are treated with a combination therapy with carbidopa, L-dopa, and 5-hydroxytryptophan additionally to a phenylalanine restricted diet and administration of BH_4 (Ponzone et al. 2006).

GTP cyclohydrolase I (GTPCH) deficiency

GPTCH deficiency (OMIM 233910), also termed arGTPCH due to its autosomal recessive trait, is a rare form of HPA, characterised by low urine and blood concentrations of pterins as the BH_4-biosynthesis is blocked at an early step in the pathway. Accordingly there are very low levels of neopterin, biopterin, isoxanthopterin and pterin detectable in the urine, but the ratio of the different pterins is normal. Also in the CSF, neopterin and biopterin, together with neurotransmitter precursors (5HIAA and HVA), are found in very low concentrations. The clinical picture of GTPCH deficiency is variable, but patients share some common symptoms like mental retardation, convulsions, disturbance of tone and posture, abnormal movements, hypersalivation and swallowing difficulties (Blau et al. 2001; Blau 2006).

6-Pyruvoyl-tetrahydropterin synthase (PTPS) deficiency

Patients with a PTPS deficiency (OMIM 261640) cannot convert dihydroneopterin triphosphate (NH_2TP), the substrate of PTPS, to 6-pyruvoyl-tetrahydropterin (6PTP), which leads to an accumulation of the substrate. In the tissue, NH_2TP is dephosphorylated by pyrophosphatases to dihydroneopterin and cleared from the body in high concentration via urine, where it can be detected, respectively its oxidation product neopterin, together with monapterin and 3'-hydroxysepiapterin. Biopterin is present only in traces. PTPS is the most frequent and heterogeneous BH_4-deficiency. Untreated patients show generally plasma phenylalanine concentration of approximately 1200 µM. About 80% of the PTPS-deficient present with a severe, or typical form. Their neurotransmitter levels are similarly low as in patients with GTPCH deficiency, in contrast to patients with a mild (atypical, peripheral/partial) variant, whose concentration of neurotransmitter metabolites in the CSF appears to be normal. But neopterin levels are increased as well (Blau et al. 2001; Blau 2006).

Pterin-4α-carbinolamine dehydratase (PCD) deficiency

PCD deficiency, also known as primapterinuria (OMIM 126090 and 264070) was initially often misdiagnosed as a mild form of PTPS deficiency. Newborns show variably elevated phenylalanine levels, which may increase transiently to concentration between 1200 µM and 2000 µM. Biopterin levels in the urine are in the subnormal range but neopterin is higher than normal values. Remarkable is that about 30% to 50% of the total biopterin are excreted as 7-biopterin (or primapterin), the substance that clearly distinguishes PCD from PTPS deficiency (Blau et al. 2001; Blau 2006).

Dihydropteridine reductase (DHPR) deficiency

DHPR deficiency (OMIM 261630) is characterised by excretion of extremely high concentrations of total biopterin, but in the newborn period urinary pterins pattern can be completely normal. The enzyme DHPR is responsible for the conversion of the highly unstable intermediate quinoid dihydrobiopterin (qBH_2), formed via hydroxylation from BH_4 through PCD, to BH_4. In cases where this enzyme is defective, qBH_2 readily tautomerises to 7,8-

dihydrobiopterin which is excreted in the urine as its oxidised product biopterin in very high quantities together with normal to slightly increased neopterin. Additionally DHPR deficient patients suffer from a defective folate metabolism in the CNS.

Most patients with such a deficiency are detected through screening of the urinary pterins, but some neonates can be missed, especially when under low-phenylalanine diet. Therefore, when DHPR deficiency is suspected, an additional DHPR enzyme assay, using dried blood spots, is performed to confirm (Blau et al. 2001; Blau 2006).

Tetrahydrobiopterin (BH_4) deficiencies without hyperphenylalaninemia (HPA)

Historically, defects of BH_4 metabolism have been identified as a cause of follow-up studies to unravel the reason of an HPA found during newborn screening or in the course of clarification of neurological signs of unclear origin in older children. The latter is especially true for a number of BH_4-deficiencies presenting without HPA, namely dopa-responsive dystonia (DRD, an autosomal dominant adGTPCH deficiency), and sepiapterin reductase (SR) deficiency (Blau et al. 2001; Blau 2006).

Dopa-responsive dystonia (DRD)

Dopa-responsive dystonia (OMIM 128230) also known as autosomal dominantly inherited GTP cyclohydrolase I (adGTPCH) deficiency or Segawa disease is characterised, as its name suggests, by a fast response to low dose L-dopa. Patients show often diurnal variation of dystonia getting worse towards the evening. First symptoms generally affect the posture of the feet. Within several years, the muscle dystonia is spreading to the other extremities including further symptoms like parkinsonism (Segawa et al. 2003).

Most informative biochemical measurements come from analysis of neopterin and biopterin, and neurotransmitter metabolites like HVA and 5HIAA in the CSF. Compared to arGTPCH deficiency, CSF levels of neopterin and biopterin are higher; nevertheless patients of both forms of a GTPCH deficiency can be clearly distinguished from patients with other types of a BH_4 deficiency or controls.

Analysis of large DRD pedigrees revealed a highly variable expressivity in clinical symptoms despite of the same mutation. Asymptomatic carriers are more frequently men than women, and the penetrance in patients was reported to be 90-100% in females and 40-55% in males, potentially due to sexual differentiation of mesencephalic dopaminergic neurons (Blau et al. 2001; Blau 2006; Ichinose and Nagatsu 2006).

Sepiapterin reductase (SR) deficiency

The autosomal recessively inherited SR deficiency (OMIM 182125) is the latest detected BH_4-deficiency affecting a different enzyme of the BH_4 metabolism and was first described by Bonafé et al. in 2001. In contrast to other BH_4-deficiencies it cannot be detected by the neonatal screening for PKU, as it presents without hyperphenylalaninemia, also urinary pterin excretion is normal. Analysis of CSF neurotransmitter metabolites and pterins, e.g. sepiapterin, as well as enzyme activity in fibroblasts are used for the diagnosis of this disorder. The discovery of SR deficiency led to new insights into alternative pathways of the cofactor BH_4, its clinical features comprise common but variable symptoms, seen also in other variants of autosomal recessive BH_4 deficiencies like disturbed tonus and posture, abnormal movements, hypersalivation, and swallowing difficulties. Besides of progressive psychomotor retardation, spasticity, and dystonia; microcephaly, tremor, seizures, and other neurological signs can be observed (Blau et al. 2001; Blau 2006; Bonafé et al. 2001; Bonafé 2006).

Table 1.
Laboratory parameters found in patients with various forms of PKU and BH_4-deficiency

Disorder	Phe (blood)	Neo (urine)	Bio (urine)	Neo (CSF)	Bio (CSF)	5HIAA (CSF)	HVA (CSF)	5MTHF (CSF)
	µM	mM/mol creat.		nM				
PKU	>1200	1.2-19.8	0.5-7.9	9-118	15-143	14-471	47-174	n
Mild PKU	600-1200	1.2-14.5	0.6-5.3	9-118	15-143	n	n	n
MHPA	120-600	<0.2	<0.2	n	n	n	n	n
arGTPCH	120-1200	<0.2	<0.2	0.05-3.0	1.5-7.5	61-183	15-48	n
PTPS	250-2500	5.0-51.2	<0.5	47-402	1.0-16.0	5-113	5-223	n
Mild PTPS	240-2200	5.0-51.2	<0.5	25-230	13-56	n	n	n
PCD	180-1200	4.1-22.5	0.7-1.5*	43-117	16-96	n	n	n
DHPR	180-2500	0.5-23.2	3.8-25.6	11-70	43-117	4-75	19-204	↓
Mild DHPR	280-600	0.5-23.2	3.8-25.6	11-70	43-117	21-66	n	↓-n
DRD	n	n	n	1.1-6.2	3.1-7.6	48-97	120-239	n
SR	n	n	n	14-51	72-102**	3-15	49-111	n
Controls	<120	1.0-15.5	0.5-7.6	15-35	20-70	310-1100	150-800	64-182

*Primapterin in urine ↑; **7,8-dihydrobiopterin and sepiapterin in CSF ↑; n = normal; PKU = phenylketonuria; MHPA = mild hyperphenylalaninemia; arGTPCH = autosomal recessive GTP cyclohydrolase I deficiency; PTPS = pyruvoyl-tetrahydropterin synthase deficiency; PCD = pterin-4α-carbinolamine dehydratase deficiency; DHPR = dihydropteridine reductase deficiency; DRD dopa-responsive dystonia; SR = sepiapterin reductase deficiency; Phe = phenylalanine; Neo = neopterin; Bio = biopterin; 5HIAA = 5-hydroxyindolacetic acid; HVA = homovanillic acid; 5MTHF = 5-methyltetrahydrofolic acid; CSF = cerebrospinal fluid.
Adapted from (Blau 2006).

Tetrahydrobiopterin metabolism

(6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (tetrahydrobiopterin, BH_4), the molecule that links PKU and BH_4-deficiencies, is crucial for several processes in the organism and is ubiquitously found in all tissues. It is either produced by *de novo* biosynthesis or via regeneration of pterin-4α-carbinolamine (4α-hydroxy-tetrahydrobiopterin), the oxidation product of different aromatic amino acid hydroxylases (AAAH) (Fig.1). The *de novo* biosynthesis starts with the conversion of guanosine triphosphate (GTP) to NH_2TP by GTPCH, followed by a redox reaction and a dephosphorylation carried out by PTPS. This reaction leads to 6PTP, which is then reduced to the final product BH_4 by SR. The biosynthesis of BH_4 via the two intermediates is a Mg^{2+}-, Zn^{2+}-, and NADPH-dependent reaction.

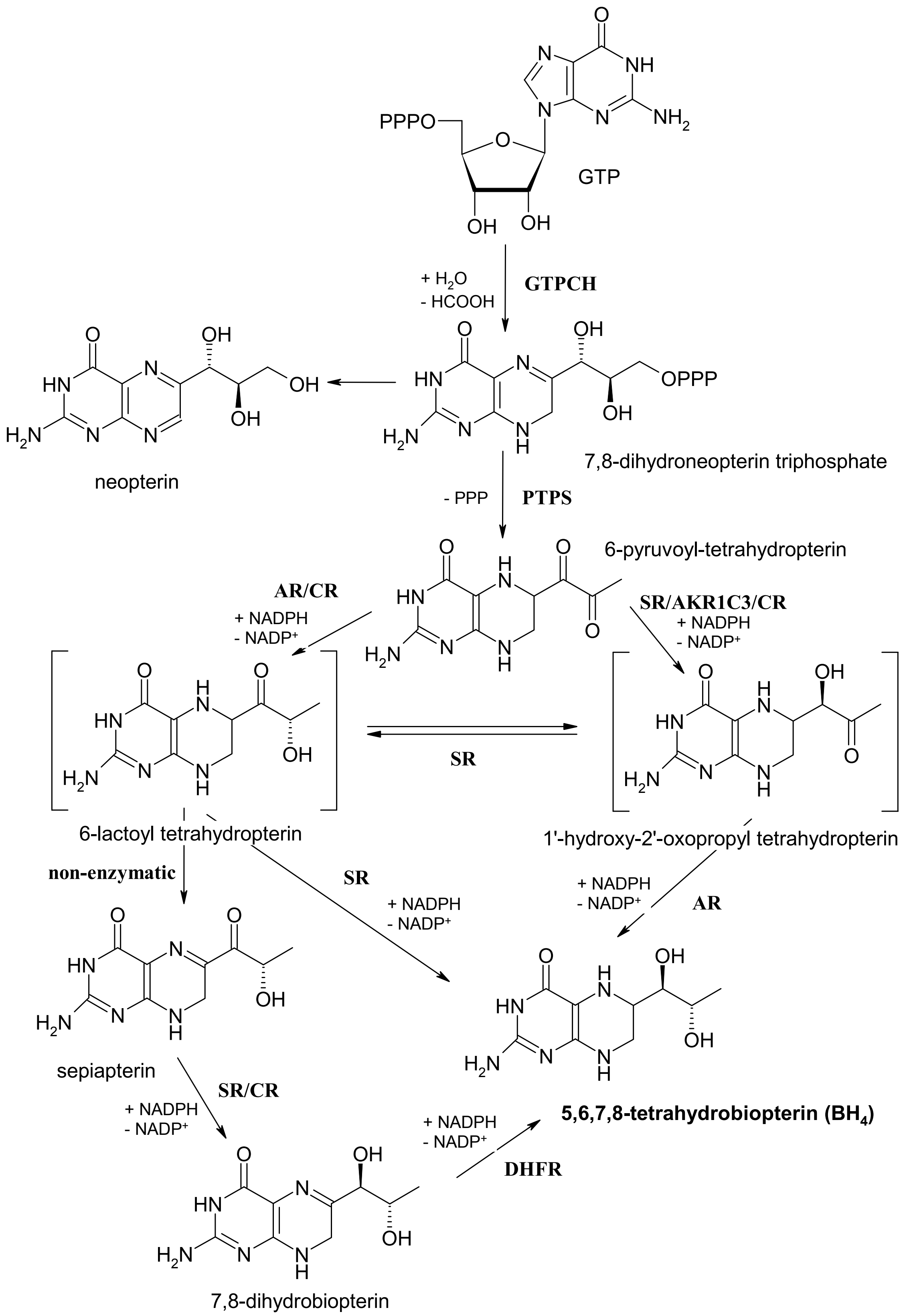
Subsequently BH_4 is primarily consumed by aromatic amino acid hydroxylases, i.e. PAH, TH;, and TPH (TPH1, peripheral and TPH2, neuronal). Furthermore, BH_4 serves as cofactor for all three forms of nitric oxide synthase (NOS; nNOS, neuronal; iNOS, inducible; eNOS, endothelial; EC 1.14.13.39) and for the enzyme glyceryl-ether monooxygenase (GEMO; EC

1.14.16.5). Besides functioning as a cofactor, BH_4 has several less defined functions at the cellular level (see below, page 22).

As mentioned before, when used as a cofactor, BH_4 is converted to pterin-4α-carbinolamine, which is thereafter dehydrated in the first step of the regeneration to quinoid-dihydrobiopterin. This reaction is catalysed by the enzyme PCD/DCoH. And finally, under consumption of NADH, q-dihydrobiopterin is converted back to BH_4 by the action of DHPR.

The regulation of the BH_4 biosynthesis, controlled by GTPCH feedback regulation protein (GFRP) and other factors, appears to be complex (see below), and a complete picture of the pathway control does not exist yet.

In the further paragraph the different enzymes, involved in the *de novo* biosynthesis and the regeneration of BH_4, will be closer portrayed.



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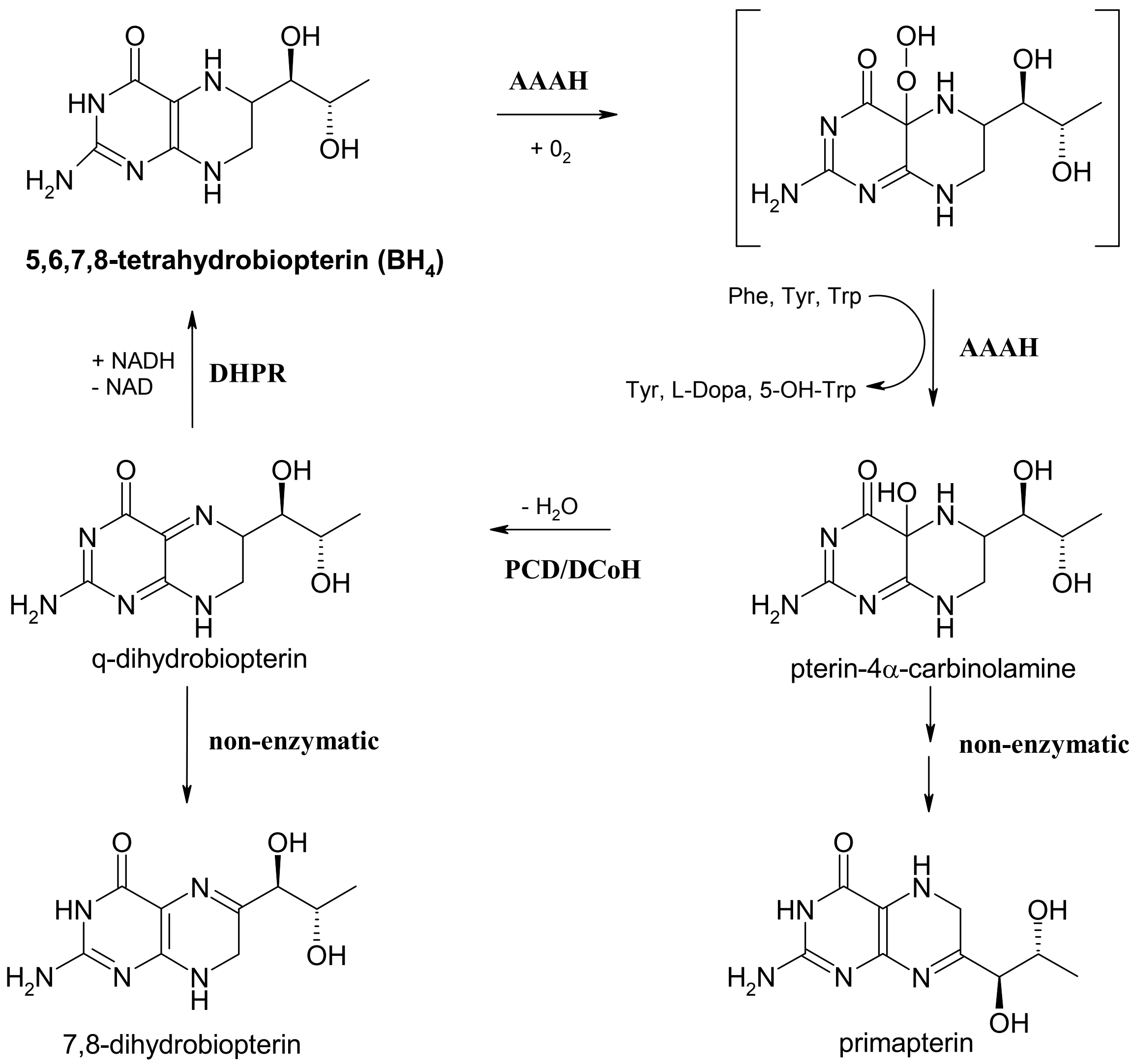

Fig. 1. BH_4-biosynthesis, consumption by aromatic amino acid hydroxylases (phenylalanine-, tyrosine-, tryptophan-hydroxylase) or similarly by nitric oxide synthase and glyceryl-ether monooxygenase, and regeneration of BH_4. Alternative pathway and salvage pathway are as well integrated into figure 1. For explanation see paragraph *Alternative and salvage pathway* (below).
GTPCH = GTP cyclohydrolase I; PTPS = pyruvoyl-tetrahydropterin synthase; SR = sepiapterin reductase; AKR1C3 = aldo-keto reductase family 1, member C3; CR = carbonyl reductase (CBR); AR aldose reductase (AKR1B1); DHFR = dihydrofolate reductase; AAAH = aromatic amino acid hydroxylase, Phe = phenylalanine; Tyr = tyrosine; Trp = tryptophan; L-dopa = levodopa (3,4-dihydroxy-L-phenylalanine); 5-OH-Trp = 5-hydroxy-L-tryptophan; PCD/DCoH = pterin-4α-carbinolamine dehydratase/dimerisation cofactor of HNF1-α; DHPR = dihydropteridine reductase.
Adapted from (Blau et al. 2002; Thöny 2006)

GTP cyclohydrolase I

The enzyme GTP cyclohydrolase I (GTPCH; EC 3.5.4.16), the first enzyme in the biosynthesis of BH_4 (Fig. 2.), is encoded by a single copy gene, *GCH1*, containing 6 exons, spanning about 30 kb and is located on chromosome 14 (14q21.1-22.2) (Ichinose et al. 1995). Its expression can be modulated on a transcriptional level by various stimuli of the immune system such as cytokines (interferon-γ, tumour necrosis factor-α, stem-cell factor, interleukin-1β, glial cell line-derived neurotrophic factor GDNF and specific combinations of these), phytohemagglutinin, and endotoxin (lipopolysaccharide) in a cell- and tissue-specific manner. Furthermore in some human cell lines (HUVEC) increased levels of *GCH1* mRNA were also observed after stimulation with phenylalanine, and after application of arginine or H_2O_2 (endothelial cells) (Thöny 2006; Werner-Felmayer et al. 2002). Post-translational modification of GTPCH include cleavage of the 11 N-terminal amino acids (shown in rats) and phosphorylation of the enzyme, most likely through protein kinase C (PKC) or casein kinase II (at least *in vitro*). Phosphorylation increases the enzymatic activity of GTPCH and leads to higher cellular BH_4 levels (Hesslinger et al. 1998; Lapize et al. 1998; Thöny 2006). Further effectors controlling the enzyme activity are, GTP – the substrate of GTPCH – and the pathway end-product, BH_4 (together with other reduced pteridines), via a negative feedback regulation involving GFRP (see paragraph below) as well as phenylalanine (positive feedback regulation via GFRP), and intracellular Ca^{2+} influx, which was shown to up-regulate GTPCH expression in cell culture models.

The enzyme GTPCH is functionally active as a homodecamer, with monomers of 250 amino acids and of 27.9 kDa. Crystal structure data is available for several organisms (recombinant human, *E. coli* and rat), and for the GTPCH·GFRP complex of rat protein (Auerbach et al. 2000; Maita et al. 2002, 2004; Nar, Huber, Meining, et al. 1995; Nar, Huber, Auerbach, et al. 1995). The homodecameric enzyme is composed of two dimeric pentamers lying face-to-face building a barrel with a cavity of 30 Å ×30 Å ×15 Å. There are ten equivalent active sites with a complexed Zn^{2+} ion per functional unit (Thöny 2006).

GTPCH feedback regulation protein

GTPCH feedback regulation protein (GFRP) is the product of the gene *GCHFR*, which is located on chromosome 15q15. It spans roughly 4 kb and codes for 3 exons. Expression analysis showed that up to three different transcripts are produced in all investigated human tissues, including brain (Gesierich et al. 2003; Milstien et al. 1996; Werner et al. 2002).

The 84-amino-acid protein has a weight of 9.7 kDa and forms a propeller-like homopentameric disc. Two such pentamers sandwich one GTPCH decamer and build a 360 kDa GTPCH·GFRP complex; the contact between GTPCH and GFRP is mediated mainly by van der Waals interactions and salt bridges. The association between the two is enhanced by binding of five phenylalanine molecules at the contact surface. This seems to increase the GTPCH activity by locking the enzyme in an active state (Thöny 2006).

6-pyruvoyl-tetrahydropterin synthase

6-pyruvoyl-tetrahydropterin synthase (PTPS; EC 4.6.1.10) is expressed from the gene *PTS* which spans a region of about 6-7 kb on chromosome 11q22.3-23.3 (Thöny et al. 1992). It contains six exons, whereof 23 bp of the exon 3 are occasionally skipped at least in some cell types. This splicing polymorphism, causing aberrant protein expression, was not only observed in patients but also in normal controls. The amount of exon-3-skipping could be closely correlated with PTPS activity, leading to the conclusion that this is a major mechanism to regulate protein expression in different human cell types (Leitner et al. 2003). Furthermore phosphorylation of PTPS was shown to be essential for the activity of the enzyme (Oppliger et al. 1995)

PTPS forms homohexamers, consisting of 2 trimers which are arranged in a head-to-head fashion building a barrel of the overall dimensions of 60 Å ×60 Å ×60 Å. The complex harbours six putative active sites, one per subunit. The monomer consists of 145 amino acids, weighing 16.4 kDa, and binds a Zn^{2+}-ion, crucial for catalysis, coordinated by three histidine residues (Ploom et al. 1999; Thöny 2006).

Sepiapterin reductase

Sepiapterin reductase (SR; EC 1.1.1.153) located on chromosome 2p13 and encoded by the gene *SPR* spans a region of approximately 4-5 kb with three exons. The genomic organisation of this gene is very similar in mouse and human and they are highly homologous (Lee et al. 1999; Ohye et al. 1998).

SR, a member of the short-chain dehydrogenase/reductase family, forms homodimers out of 261-amino-acid, and 28.0 kDa monomers, and seems ubiquitously expressed in all mammalian tissues, also throughout all brain regions in contrast to GTPCH or PTPS which are mainly expressed in monoamine neurons (Thöny 2006). *In vitro* experiments showed that SR is phosphorylated by calmodulin-dependent protein kinase II and by protein kinase C.

Phosphorylation changed the kinetic properties and might also play a role in the regulation of SR in vitro (Fujimoto et al. 2002; Katoh et al. 1994).

Pterin-4α-carbinolamine dehydratase

Pterin-4α-carbinolamine dehydratase (PCD; EC 4.2.1.96) is also known as pterin-4α-carbinolamine dehydratase/dimerisation cofactor of hepatocyte nuclear factor 1-α (HNF1-α) or short: PCD/DCoH. PCD is encoded by the genes *PCBD1* on chromosome 10q22, spanning four exons (Citron et al. 1993; Thöny et al. 1994, 1995) and as a paralogue enzyme (also known as DCoH2) by *PCBD2* located on chromosome 5q31.1, equally spanning 4 exons (http://www.ncbi.nlm.nih.gov/ Entrez gene) (Rose et al. 2004).

A PCD monomer weighs 11.9 kDa (104 amino acids) but the functional enzyme with dehydratase activity consists in a homotetramer with an overall size of 60 Å ×60 Å ×60 Å. Primary sequence of the mature protein is identical in human and rat, and differs only by one amino acid from the one of the mouse. The mammalian PCD/DCoH and DCoH2 exist in two oligomeric states; in the cytoplasm they adopt a homotetrameric conformation with dehydratase activity, and in the nucleus they can be found as an $\alpha_2\beta_2$ heterodimer associated with HNF1-α, enhancing thereby as a coactivator the transcriptional activity of HNF1-α. DCoH2 can only partially complement PCD/DCoH activity in the regeneration of BH_4, but the two paralogues are able form homotetramers and mixed heterotetramers in solution.

PCD activity was found in human liver, kidney, brain, skin and hair follicles. Patients with the chronic skin condition vitiligo showed dramatically reduced PCD activity (Schallreuter 1999; Thöny 2006).

Dihydropteridine reductase

Dihydropteridine reductase (DHPR; EC 1.6.99.7) is encoded by the gene QDPR, located on chromosome 4p15.3. This 7-exon gene spans over more than 20 kb, the coding sequence consisting of 732 bp.

The protein counts 244 amino acids, leading to a molecular weight of 25.8 kDa. DHPR forms dimers of a dimension of 34 Å ×50 Å ×73 Å. Crystal structure is available for the rat protein at a 2.3 Å resolution (Varughese et al. 1992). DHPR is an α/β protein with a Rossmann-type dinucleotide fold for NADH binding; the human enzyme was found to bind two NADH molecules per dimer.

DHPR has an essential role in the hydroxylation systems of phenylalanine, tyrosine, and tryptophan and is widely distributed in the tissue. In addition to its expression in the adrenal medulla, the principal site of the conversion of tyrosine to catecholamines, and the brain (conversion of tyrosine and tryptophan in the biosynthesis of the neurotransmitters serotonin and dopamine), it is also found in tissues with no or only little aromatic amino acid hydroxylation activity, like in the heart or the lung. Its function in those tissues is unclear but it might be involved in other metabolic processes (Blau et al. 2001; Thöny 2006).

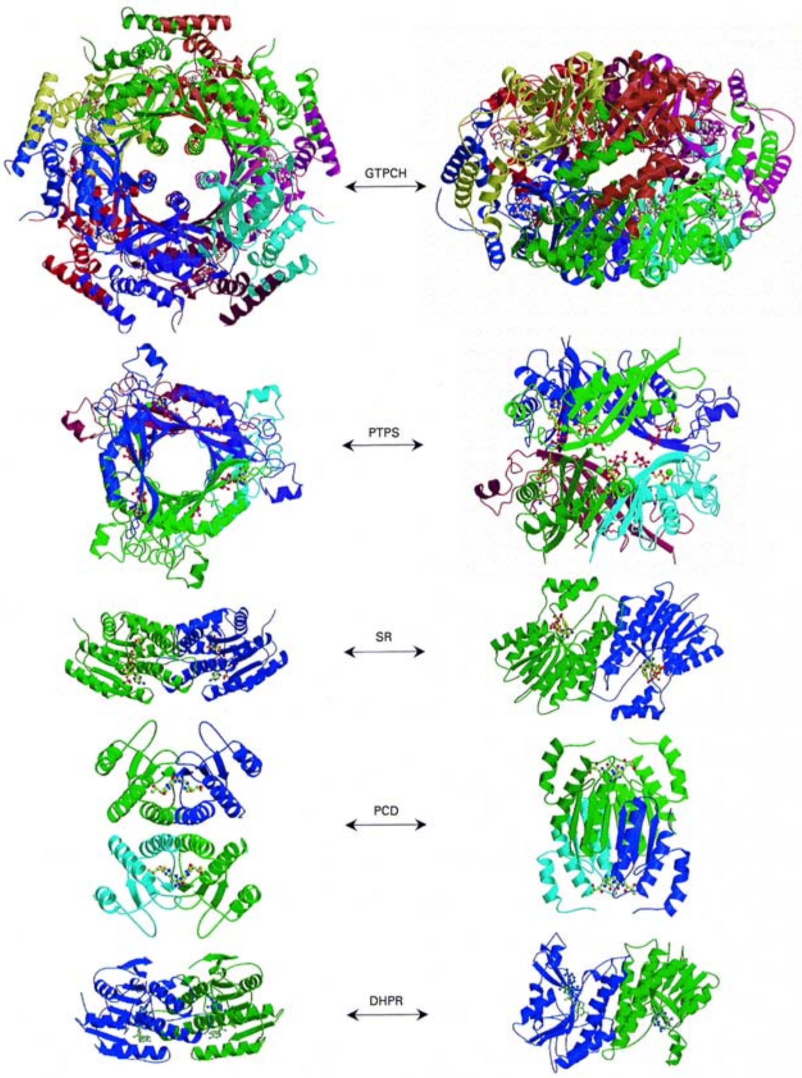

Fig. 2. Three-dimensional structures of BH_4-metabolising enzymes. Shown are ribbon-type representations of the main-chain foldings of the enzymes involved in the *de novo* BH_4-biosynthesis and BH_4-regeneration. Substrates are shown in ball-and stick representation, with atoms in standard colours. On the right side the enzymes are shown rotated by 90° around the x-axis. The crystal structure co-ordinates used are: GTPCH from *E. coli*, PTPS from rat liver, SR from mouse, PCD/DCoH from rat, and DHPR from rat liver. GTPCH = GTP cyclohydrolase I; PTPS = pyruvoyl-tetrahydropterin synthase; SR = sepiapterin reductase; PCD = pterin-4α-carbinolamine dehydratase; DHPR = dihydropteridine reductase. Figure taken from (Thöny et al. 2000).

Alternative and salvage pathway

Besides the *de novo* biosynthesis and the regeneration by PCD and DHPR, BH_4 can also be produced through the so-called salvage pathway involving the enzyme SR (Fig. 1); i.e. SR catalyses the conversion of sepiapterin to 7,8-dihydrobiopterin which is subsequently reduced to

BH_4 by dihydrofolate reductase (DHFR; EC 1.5.1.3), in both steps NADPH is consumed (Nichol et al. 1985). Even though SR is enough to complete the BH_4 synthesis, there exist alternative pathways for the production of BH_4 and especially the detection of the SR deficiency, due to its lack of hyperphenylalaninemia provided, deeper insight into these pathways of BH_4 metabolism. It involves a family of NADPH-dependent aldo-keto reductases, and carbonyl reductases (CR), aldose reductases and the 3α-hydroxysteroid dehydrogenase type 2 (AKR1C3). They are able to convert 6-pyruvoyl-tetrahydropterin via different routes to BH_4 in the interaction with SR but also without. Moreover CR is able to catalyse the conversion of sepiapterin to 7,8-dihydrobiopterin like the SR (Iino et al. 2003). It is assumed that due to a low expression or activity of DHFR and AKR1C3 in the brain, the BH_4 synthesis starting from 6-pyruvoyl-tetrahydropterin via alternative pathway is not sufficient in the case of a SR deficiency and leads to a central BH_4-deficiency without HPA.

Furthermore, both sepiapterin and 7,8-dihydrobiopterin can be taken up and metabolised by the salvage pathway to replenish the BH_4 pool in the body. Studies with mice showed that these two compounds are taken up even more efficiently than natural BH_4 (Blau et al. 2001; Sawabe et al. 2005; Thöny 2006).

Tetrahydrobiopterin as a cofactor and other functions

The history of BH_4, respectively of pterins, dates back to the end of the 19^{th} century, when in 1889 the later Nobel Laureate and butterfly fancier Sir Frederick Gowland Hopkins partially characterised a yellow purine-like pigment from the wings of the common English brimstone butterfly and some years later, a white pigment from the wings of the cabbage butterfly, now known as xanthopterin and leucopterin (Curtius 2006). In 1963 Pfleiderer proposed the trivial name *pterin* for the compound 2-amino-1H-pteridin-4-one and its derivatives and in same year Kaufman recognised BH_4 as the natural cofactor of PAH (Curtius 2006; Kaufman 1963).

To date, the function of BH_4 as cofactor for the aromatic amino acid hydroxylases PAH, TH, and TPH1, TPH2 is the most investigated and understood. Furthermore it is used by all three forms of the nitric oxide synthase and the enzyme glyceryl-ether monooxygenase (Kappock and Caradonna 1996; Taguchi and Armarego 1998; Werner et al. 2007).

Enzymatic reactions of AAAH have intensively been studied and revealed a strict requirement not only for BH_4 but also for O_2, and iron-ions. The oxidation product of BH_4, tetrahydro-4α-carbinolamine, is released after each catalytic turnover to be regenerated by PCD and DHPR. In the course of the reaction two electrons are transferred from BH_4, one to reduce the iron from Fe^{3+} to Fe^{2+} and the second goes apparently to the oxygen (Hufton et al. 1995). In contrast to this, the role BH_4 plays in reactions of NOS seems to be a different. BH_4 appears not to dissociate from NOS during the turnover and only a one-electron transfer takes place.

Besides serving as a cofactor for PAH, BH_4 together with phenylalanine has a major regulatory function for this enzyme. BH_4 works a negative effector, forming a dead-end complex PAH·BH_4; on the other hand phenylalanine is a positive allosteric effector, competing with BH_4 to turn the inactivated enzyme into an enzymatically active state. In the liver, the site of the highest PAH activity, BH_4 concentrations were found to be around 5-10 µM, but the K_m value for the cofactor of PAH was estimated to be approximately 20-30 µM, leaving BH_4 in sub-saturating concentrations and making it a limiting factor in the catabolism of phenylalanine in the liver (with important implications for BH_4-responsive HPA/PKU). Recently it was recognised that BH_4 has an additional protecting role for PAH as a chemical chaperone, preventing the protein from misfolding and inactivation during dimer/tetramer formation and protecting it against proteolytic cleavage (Pey et al. 2004; Thöny et al. 2004).

All three isoforms of the NOS use BH_4 as redox cofactor; each monomer has a C-terminal reductase domain, where NADPH, FAD, FMN, and calmodulin can bind and an N-terminal oxygenase domain with binding sites for the cofactor BH_4, the prosthetic group heme and for the substrate arginine. But NOS are functionally active as dimers and carry therefore two molecules of BH_4 at the interface between the two monomers. BH_4 is not only required for the catalysis serving as a one-electron donor in the conversion of arginine to citrulline but has also structural functions, including stabilisation of the NOS dimer, and protecting it against proteolysis. Additionally BH_4 facilitates the binding of arginine (Stuehr et al. 2004; Wei et al. 2003). Compared with PAH, NOS' need for BH_4 is, according to its K_m value, two orders of magnitude smaller. Nevertheless, when the concentration drops too low, as it might be the case in BH_4 deficiency, BH_4 becomes a limiting factor for the activity of NOS, which becomes uncoupled and produces peroxides instead of nitric oxide; furthermore the available NO can react with superoxide to form neurotoxic peroxinitrate (Zorzi et al. 2002).

Besides serving as a cofactor, BH_4 has different functions in humans but also in other organisms. One of the earliest discovered was its role as growth factor for *Crithidia fasciculata.* This fact was initially used to measure biopterin in different body fluids and tissues (Leeming and Blair 1974). BH_4 has also a proliferative activity in haematopoietic cells as newer observations do indicate (Kerler et al. 1989; Tanaka et al. 1989). DNA synthesis was found to be stimulated by BH_4 in some mouse erythroleukemia clonal cell line. In these cells proliferation was induced, as it was the case SV40 transformed human fibroblasts, rat C6 glioma cells, and PC12 cells. As sepiapterin is readily taken up by cells and converted to BH_4, it has similar effects on the cells like BH_4. Further involvement of BH_4 comprises an increased proliferation of PC12 cells through epidermal growth factor, nerve growth factor, and insulin-like growth factor.

BH_4 not only plays a role in proliferation but also seems to act as a self-protecting factor for nitric oxide toxicity as it shows a strong scavenging activity against superoxide anion radicals. On the base of this, it was suggested application of BH_4 might be a useful treatment of various diseases, which pathogenesis is actively oxygen-related (Kojima et al. 1995). Others observed that in cytotoxicity due to nitric oxide involving H_2O_2 production and scavenging of H_2O_2 by BH_4 may be at least one of the mechanisms by which BH_4 reduces NO-induced endothelial cell death (Shimizu et al. 1998). On the other hand, it was suggested that ischemia raises the intracellular BH_4 concentration and that the increased BH_4 has a pivotal responsibility in selective neuronal injury via NOS activation. They showed a distinct reduction of BH_4 concentrations accompanied by a delayed neuronal injury in the parts of the hippocampus by using a selective GTPCH inhibitor in an animal model exposed to transient forebrain ischemia (Cho et al. 1999). A further function of BH_4 is enhancing dopamine- and serotonin-release in the striatum and frontal cortex as shown in rats (Wolf et al. 1991; Koshimura et al. 1990).

Recent findings suggested that BH_4 is also a key modulator of peripheral neuropathic and inflammatory pain through the action of GTPCH. After axonal injury, concentrations of BH_4 rose in primary sensory neurons, and in dorsal root ganglia. Inhibiting the *de novo* BH_4 synthesis in rats attenuated neuropathic and inflammatory pain and prevented excess nitric oxide production in the ganglia evoked through nerve injury (Tegeder et al. 2006).

The role of BH_4 was also discussed in connection with cofactor administration in BH_4-responsive HPA/PKU patients. For these patients BH_4 presents a possible alternative to the phenylalanine restricted diet treatment. Recent studies on the mechanism of the BH_4-responsiveness unravelled a multifactorial base, including the chemical chaperon effect of BH_4, preventing inactivation and degradation of mutant PAH protein or providing certain K_m-mutants with sufficient BH_4-cofactor. PAH enzyme activity was increased by BH_4 but neither gene expression nor mRNA stability of PAH was changed in feeding experiments with BH_4 in normal mice (Kure et al. 1999; Scavelli et al. 2005; Thöny 2006; Thöny et al. 2004).

Aims of the thesis

In the light of BH_4 becoming a potential pharmacological treatment in PKU and already being used in the medication of BH_4 deficient patients, we were interested in the effects of exogenous BH_4 or increased BH_4 biosynthesis on the organism. Therefore, we started a project aiming to study the BH_4 metabolism and regulation, and the influence of BH_4 on other genes and proteins, either directly participating in BH_4 biosynthesis and regeneration or otherwise involved in the BH_4 metabolism; and the potential influence of BH_4 on other pathways. To reach our goal we intended to employ different cell models in a first approach and study the effects in patients with BH_4-responsive HPA/PKU and BH_4 deficiency.

To study effects of BH_4 orally administered to patients or healthy controls, we developed a ***new method*** assessing the pharmacokinetics of BH_4, described in **Chapter 2**, which not only provided an insight into the absorption, distribution, metabolism, and elimination time of the cofactor but also allowed for a rapid diagnosis of BH_4 deficient patients amongst newborns with hyperphenylalaninemia in the differential screening. An early onset of the correct therapy is imported for patients with hyperphenylalaninemia, therefore a method to discern the cause of the increased phenylalanine levels is essential. Until now, in screening for BH_4 deficiencies, mostly urine samples were analysed. We present here an inexpensive, easy and safe to handle alternative method, employing dried blood spots. This method was used to determine basic pharmacokinetic parameters for BH_4 in blood after administration of BH_4 or following combined phenylalanine and BH_4 loading of PAH deficient patients as described in **Chapter 3**. Extensive pharmacokinetic studies of BH_4 have been performed in animal models, but only a few parameters are known from studies in human. The ***pharmacokinetics*** was suggested being a factor affecting BH_4 loading tests and might provide details on pharmacological response to BH_4 therapy. In **Chapter 4** we aimed to identify mutations and ***genotypes*** associated with BH_4 responsiveness and estimated the dispersal of such mutations within the population of PKU patients in Europe by exploiting the BIOPKU database, an international data collection of published BH_4-responsive HPA/PKU cases, and other sources. **Chapter 5** presents a study on the outcome and long-term follow-up of patients suffering from a cofactor deficiency. These patients are under treatment with BH_4 and further medication. The aim was to recognise parameters determining a successful outcome of long-term treatment. In the final section, **Chapter 6**, preliminary results from ***gene expression*** analysis in cell models are presented. Using BH_4, respectively sepiapterin which is readily taken up by cells and converted to BH_4, and other stimulating or inhibiting agents, we manipulated and analysed the regulation of the BH_4 metabolism. To reach this goal we systematically tested several cell lines for activity of enzymes involved in the metabolism of BH_4, and a few cell lines were further investigated to study the influence of increased BH_4 concentrations on the metabolism.

Chapter 2 is published in Mol Genet Metab (Zurflüh et al. 2005)

Chapter 3 in J Inherit Metab Dis (Zurflüh et al. 2006)

Chapter 4 in Hum Mutat (Zurflüh et al. 2008)

Chapter 5 in Mol Genet and Metab (Jäggi et al. 2008)

Chapter 6 is intended for publication (Zurflüh et al. 2008)

Appendix is published in Eur J Pediatr (Opladen et al. 2005)

Chapter 2

SCREENING FOR TETRAHYDROBIOPTERIN DEFICIENCIES USING DRIED BLOOD SPOTS ON FILTER PAPER

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Abstract

Tetrahydrobiopterin (BH_4) deficiency among newborns with hyperphenylalaninemia must be rapidly diagnosed and distinguished from classical phenylketonuria (PKU) to initiate immediately specific treatment and to prevent irreversible neurological damage. The characteristic pattern of urinary pterins makes it possible to differentiate between PKU and BH_4 deficiencies, and to identify different variants of BH_4 deficiency. However, collection, storage, and shipment of urine samples for pterin analysis is cumbersome. A method for the measurement of different pterins (neopterin, biopterin, and pterin) in blood collected on filter paper was developed as a potential alternative to the screening for BH_4 deficiencies in urine and for the monitoring of BH_4 pharmacokinetics. Pterins pattern in blood spots was comparable with those in plasma and urine. We thus established reference values for pterins in blood spots in patients with hyperphenylalaninemia and identified new patients with GTP cyclohydrolase I deficiency, 6-pyruvoyl-tetrahydropterin synthase deficiency, and dihydropteridine reductase deficiency using dried blood spots on filter paper.

Introduction

Tetrahydrobiopterin (BH_4) is the essential co-factor/co-substrate of phenylalanine hydroxylases (PAH) and several other monooxygenases (Thöny et al. 2000). Measurement of pterins in different biological fluids is the most common method for the screening and differential diagnosis of inborn errors of BH_4 metabolism. Five distinct genetic defects are known to cause hyperphenylalaninemia (HPA), including the classical form of Phenylketonuria (PKU) with a defect in the apo-enzyme PAH or a defect in four out of five BH_4 co-factor-synthesizing or regenerating enzymes (Blau et al. 2001). Either of two defects in biosynthesis of BH_4, i.e., GTP cyclohydrolase I (GTPCH) or 6-pyruvoyl-tetrahydropterin synthase (PTPS) or defects in regeneration, i.e., pterin-4α-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR) may be responsible for BH_4 deficiency. BH_4 deficiency is a severe but treatable disease and early detection in newborns is essential to avoid irreversible brain damage.

According to the current protocol, the following investigations should be performed in all newborns with HPA (blood phenylalanine >120 μmol/L): 1. analysis of pterins (neopterin, biopterin, and primapterin) in urine; 2. measurement of DHPR activity in dried blood spots from Guthrie card; and 3. analysis of phenylalanine and tyrosine in plasma or blood before and after BH_4 loading with 20 mg/kg body weight. Tests 1 and 2 are essential for all newborns and identify primarily variants of BH_4 deficiency in older children due to characteristic pterin patterns: GTPCH deficiency with low neopterin and biopterin, PTPS deficiency with high neopterin and only traces of biopterin, PCD deficiency with high neopterin, moderately biopterin and high primapterin, and DHPR deficiency with normal or moderate elevated neopterin and high biopterin. Test 2 identifies patients with DHPR deficiency, which can sometimes be missed by test 1 if the urine is collected under low-protein diet. Test 3 is useful in all forms of BH_4 deficiency and can also detect patients with BH_4-responsive PAH deficiency (Blau et al. 2002).

With the introduction of tandem mass-spectrometry (TMS) in newborn screening, blood sample collection on filter paper became a routine procedure. The aim of this study was to test the use of filter paper blood spots (Guthrie cards) instead of urine for screening of BH_4 deficiencies and to study the pharmacokinetics of BH_4 by monitoring blood concentrations of pterins following oral loading test in patients with HPA. The new method should enable simultaneous measurement of pterins (neopterin, biopterin, isoxanthopterin, and pterin), DHPR activity, and amino acids from a single Guthrie card specimen. The main advantage of using Guthrie cards instead of urine is the easy handling and sample collection, and the less expensive shipping of the samples at room temperature.

Materials and Methods

Pterins were purchased from Schircks Laboratories (Jona, Switzerland). All other chemicals were of the highest quality available.

Sample preparation

Pterins were eluted from dried blood spots on filter paper (Guthrie cards). For every single measurement four blood spots (∅ 6 mm) were cut out and pterins were extracted with 250 µL of 20 mmol/L HCl and placed in an ultrasonic bath (Sonorex RK31, Bandelin) for 30 s. Extraction was continued for 10 min by mixing the filter spots solution five times for 5 s at room temperature. The extract was centrifuged at 1800*g* for 5 min at room temperature. Sixty microliters of the clear supernatant were used for analysis of haemoglobin on the haematology analyzer Sysmex KX-21N (Sysmex, Japan). The remaining supernatant was ultra-filtrated on Ultrafree (NMWL 10000; Millipore) at 5000*g* for 5 min. Pterins were analyzed in clear filtrate by HPLC and fluorescence detection without prior oxidation.

HPLC of pterins

HPLC of pterins (neopterin, biopterin, isoxanthopterin, and pterin) was performed as described previously (Curtius et al. 1991) with some modifications. Separation was performed on a C8 Spherisorb, 5 µm pre-column (40 × 4.6 mm) and ODS-1 Spherisorb, 5 µm analytical column (250 × 4.6 mm) (both from Stagroma, Rheinach, Switzerland), using 1.5 mmol/L potassium hydrogen phosphate buffer, pH 4.6, with 8% (v/v) methanol at a flow rate of 1.2 mL/min. Pterins were detected by their native fluorescence at λ_{EX}: 350 nm, λ_{EM}: 450 nm using a fluorescence Detector FP-920 (Jasco, Tokyo, Japan).

Patients

Seventy patients with HPA (age 1 week to 15 years) were either tested for BH_4 responsiveness (BH_4 loading test with 20 mg/kg) or on treatment with BH_4 (Schircks Laboratories, Jona, Switzerland). Blood sampling was a part of the routine screening for BH_4 deficiency and was approved by corresponding boards.

Controls

Healthy adult controls (authors of this work) were administered BH_4 (2 mg/kg, orally or sublingually) as described previously.

Statistical analyses

WinSTAT for Excel (v. 2003.1) was used for descriptive statistics and for regression analysis. Correlation between blood spots, plasma, and urine was evaluated by debiased regression analysis according to the method of Passing and Bablok.

Results

BH_4 is extremely unstable in collected blood and about 30–40% is readily decomposed to pterin. Thus, in this study total biopterin was calculated as the sum of biopterin and pterin (biopterin + pterin).

Extraction of pterins from filter paper

Neopterin, biopterin, and pterin were eluted from filter paper with 20 mmol/L HCl. Addition of 10 or 20% (v/v) methanol did not improve the efficiency (Fig. 1A). Extending the sonification time from 30 s to 5 min and extraction time from 10 to 30 min, and increasing the volume of solvent from 250 to 500 µL also did not change the elution profile significantly (Figs. 1B and C).

Recovery

Recovery of pterins from Guthrie cards, calculated by spiking blood samples with neopterin (20 nmol/L), biopterin (20 nmol/L), and pterin (5 nmol/L) standards, yielded 63–69%.

Stability

The stability of pterins in dried blood spots was tested by storing Guthrie cards at room temperature in the dark for a period of up to 16 days. This time was estimated to be sufficient to send samples by ordinary mail to the laboratory. Pterins were analyzed on 5 different days, and as shown in Fig. 2, the profile did not change significantly during the first 16 days. For long-time storage, Guthrie cards were kept at −20 °C and retested after 6 and 18 months. Compared with initial values, neopterin was 102%, biopterin 105%, pterin 67%, and biopterin + pterin 80% (data not shown).

Reproducibility

The run-to-run imprecision was determined with 20 identical blood samples from a patient with HPA (high value) and of an adult healthy control (low value). The rather high coefficient of variation (CV) in the control sample (low value) may be explained by the fact that concentrations of pterins were close to detection limit of the HPLC system (around 0.02 nmol/g Hb). The within-run imprecision was measured in the same fashion but samples were measured in the same series (Table 1).

Comparison with plasma and urine

Concentrations of pterins in dried blood spots were compared with those in plasma obtained at same time points from four healthy persons loaded with 2 mg BH_4/kg and regression was calculated using the method of Passing–Bablok (Table 2). A relatively good correlation was found between two methods for biopterin + pterin and neopterin (Figs. 3A and D) and less good but still acceptable for biopterin and pterin alone (Figs. 3B and C). Analysis of pterins in dried blood spots and plasma of healthy persons loaded with BH_4 (2 mg/kg) showed identical profiles for biopterin + pterin and neopterin (data not shown).

Comparison of pterins from Guthrie cards with those from urine specimen from both control persons and patients with HPA revealed a similar correlation as between blood spots and plasma (Table 2 and Figs. 4A–D). Furthermore, the percentage of biopterin + pterin (of the sum of all pterins) in two patients with HPA were found to be comparable for all three methods (Fig. 5). The first patient underwent an extended loading test with BH_4 (2 × 20 mg/kg BH_4 at T_0 and T_{24}) (Fig. 5A) and in the second patient a combined loading test (100 mg/kg phenylalanine at T_{-3} and 20 mg/kg BH_4 at T_0) was performed (Fig. 5B). The pterins profile showed that biopterin + pterin levels peaked 3–4 h after BH_4 administration in both cases (Figs. 6A and B), while neopterin concentrations remained unchanged (data not shown). The maximal BH_4 concentrations in blood spots were found to be extremely variable (3.6–96.4 nmol/g Hb) in a group of patients loaded with the same amount of BH_4 (20 mg/kg). In one patient who was, based on the genotype, suggested to be BH_4-responsive, pterins were measured in blood spots on two occasions (Fig. 7). In the first loading test, he was found to be a non-responder and at that time the blood spot biopterin + pterin concentration was rather low (9.7 nmol/g Hb). In the second test, the patient was found to be a BH_4-responder and the maximal biopterin + pterin concentration was much higher (33.2 nmol/g Hb).

Pterins profile in dried blood spots

To be able to screen for BH_4 deficiency in patients with HPA, reference values for neopterin and biopterin + pterin were established (Table 3). For this purpose, blood spots from 70 patients with various degrees of HPA in whom BH_4 deficiency was excluded by standard tests were analyzed. Values were corrected for the reported recovery of ~65%.

In a heterogeneous group of patients with HPA, two patients with PTPS deficiency, one with DHPR deficiency, and one with GTPCH deficiency were identified by analysis of the pterins profile in blood spots. Fig. 8A shows a patient suffering from classical PKU with a PAH deficiency. Neopterin and biopterin levels are slightly increased, with the profiles fitting well those in urine. A patient with a PTPS deficiency shows increased neopterin and no biopterin (Fig. 8B) and a patient suffering from DHPR deficiency has normal to increased neopterin and increased biopterin levels in blood spots (Fig. 8C). The patient with GTPCH deficiency is characterized by reduced neopterin and biopterin levels (Fig. 8D). We also investigated blood spots and urine samples from an older patient previously diagnosed with PCD deficiency, but primapterin was very low in urine and was not detected in blood spots (data not shown).

Figures and Tables

Table 1.
Run-to-run and within-run imprecision of identical 20 samples with low-value pterins (adult control) and high-value pterins (patient with HPA)

n = 20	Low value				High value			
	Neo	Bio	Pte	Bio + Pte	Neo	Bio	Pte	Bio + Pte
Run-to-run								
x	0.08	0.26	0.22	0.33	2.81	12.44	14.86	27.29
SD	0.02	0.08	0.08	0.17	0.40	2.12	2.52	4.58
CV(%)	24.0	28.4	36.0	52.9	14.3	17.0	17.0	16.8
Within-run								
x	0.05	0.21	0.41	0.62	0.32	54.23	84.69	138.92
SD	0.004	0.01	0.05	0.06	0.041	1.812	3.619	4.652
CV(%)	9.1	5.5	12.2	8.8	13.1	3.34	4.27	3.35

For details, see Materials and methods. Values are expressed in nmol/g Hb (not corrected for the recovery). Neo, neopterin; Bio, biopterin; Pte, pterin.

Table 2.
Regression analysis of pterins in blood spots, plasma, and urine according to Passing-Bablok

	n	*r*	Slope (95%-range)	Intercept (95%-range)
Blood spots vs. plasma				
Bio+Pte	72	0.883	0.006 (0.005 to 0.007)	-0.026 (-0.056 to 0.012)
Bio	72	0.773	0.004 (0.003 to 0.005)	-0.003 (-0.038 to 0.020)
Pte	72	0.728	0.010 (0.008 to 0.012)	0.000 (-0.019 to 0.037)
Neo	72	0.842	0.010 (0.009 to 0.011)	-0.025 (-0.042 to -0.012)
Blood spots vs. urine				
Bio+Pte	84	0.852	0.261 (0.217 to 0.350)	-0.062 (-0.171 to -0.021)
Bio	84	0.788	0.182 (0.152 to 0.242)	-0.019 (-0.084 to 0.016)
Pte	84	0.502	0.520 (0.341 to 0.703)	-0.005 (-0.035 to 0.000)
Neo	84	0.874	0.163 (0.151 to 0.195)	0.026 (0.009 to 0.036)

See also Figs.3 and 4.

Table 3.
Reference values for neopterin and biopterin + pterin in blood spots (nmol/g Hb) of patients with HPA, corrected for the reported recovery (65%)

n = 70	Neo	Bio+Pte	%Bio+Pte
Median	1.08	0.77	31.3
5 – 95 percentile	0.31 – 4.45	0.15 – 2.91	13.9 – 78.3

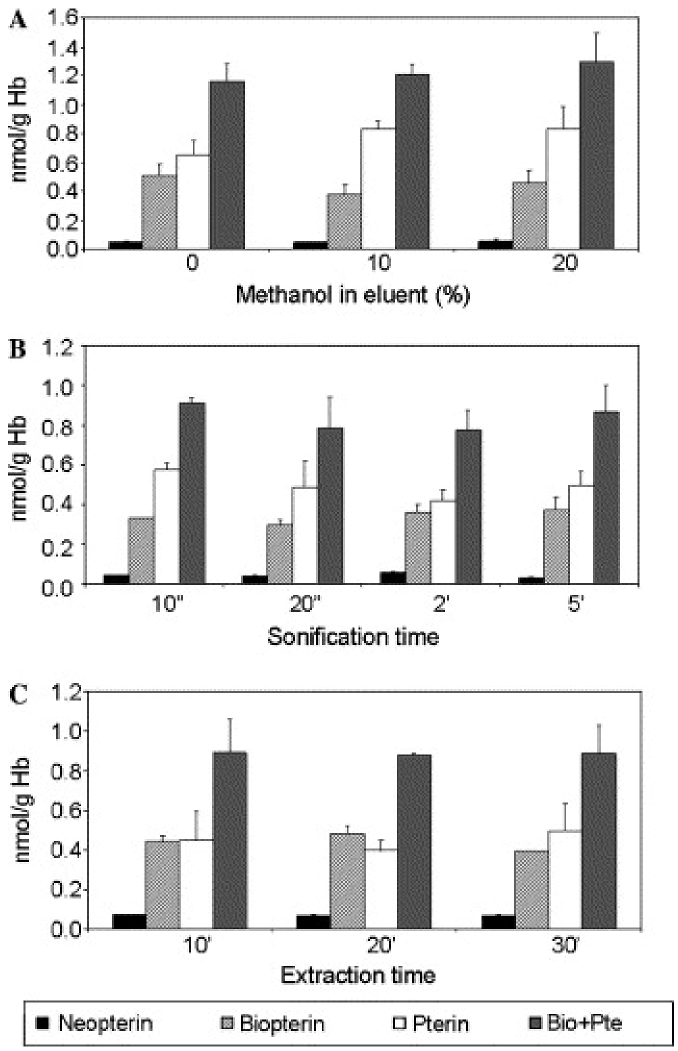

Fig. 1. Concentrations of neopterin, biopterin, and pterin in blood spots on filter paper investigated at: (A) different content of methanol in eluent; (B) different sonification times; and (C) different extraction times.

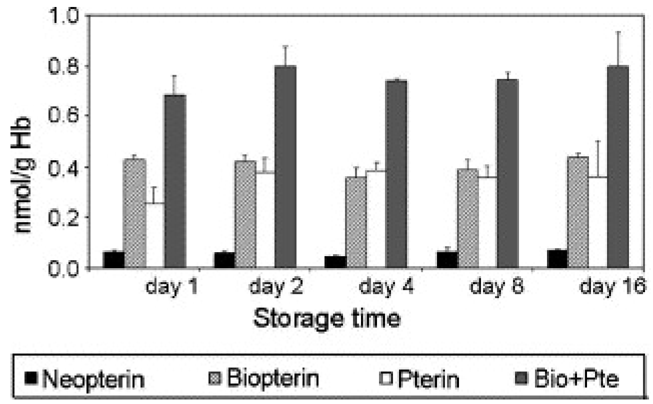

Fig. 2. Stability of neopterin, biopterin, and pterin in blood spots on filter paper after storage at room temperature. $n = 3$

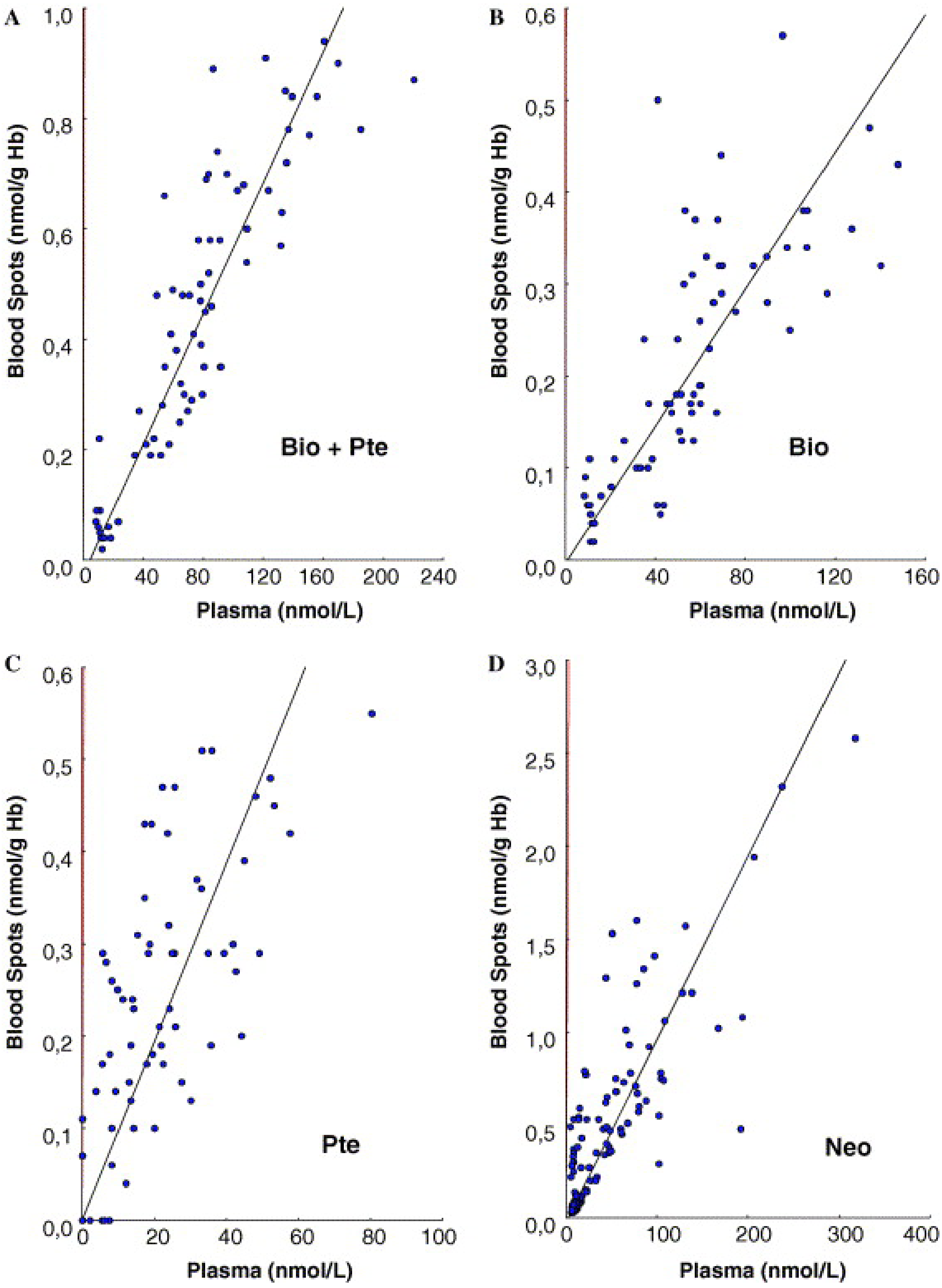

Fig. 3. Regression analysis between blood spot and plasma: (A) biopterin plus pterin (Bio + Pte), (B) biopterin (Bio), (C) pterin (Pte), and (D) Neopterin (Neo).

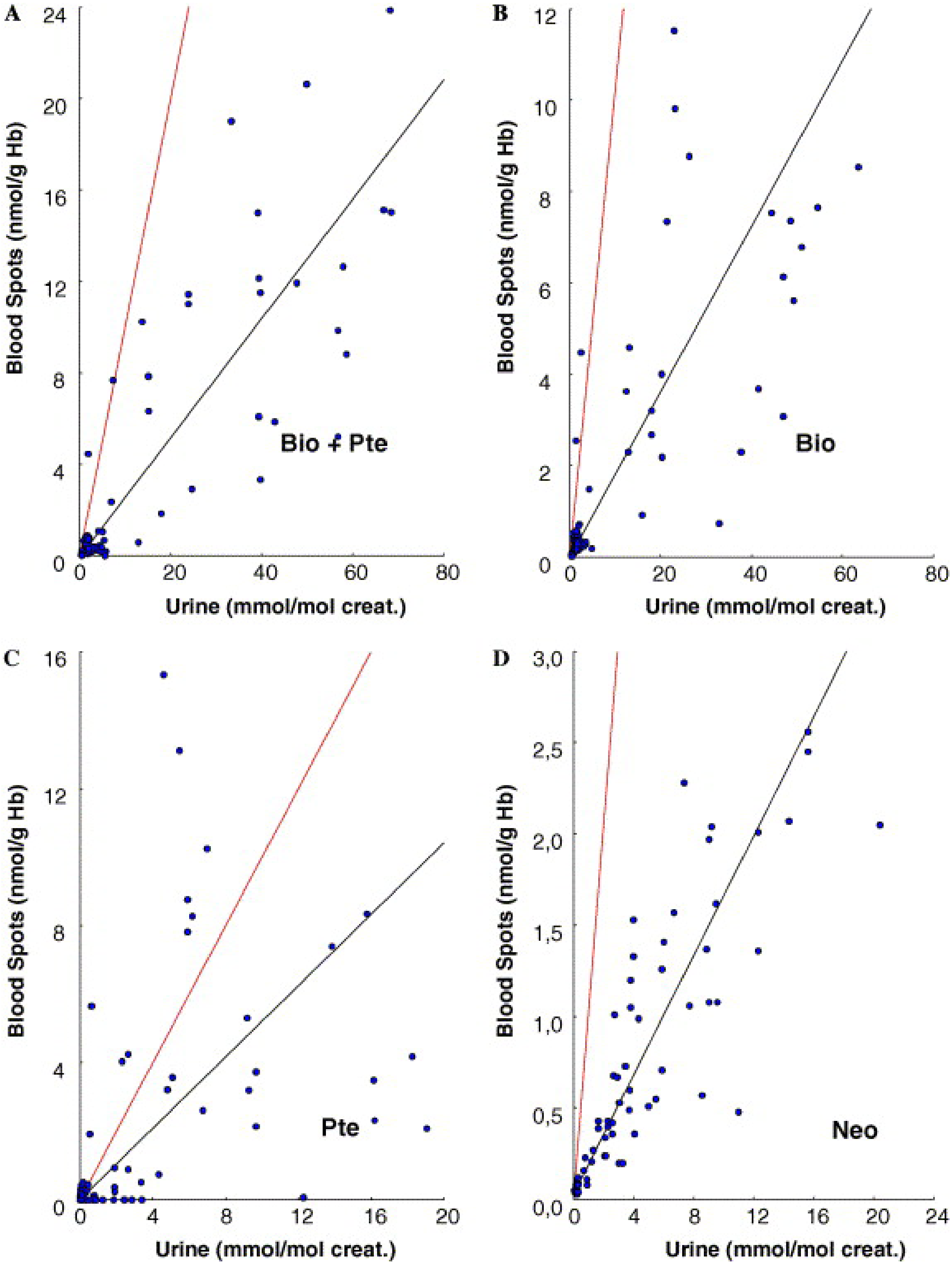

Fig. 4. Regression analysis between blood spots and urine: (A) biopterin plus pterin (Bio + Pte), (B) biopterin (Bio), (C) pterin (Pte), and (D) neopterin (Neo).

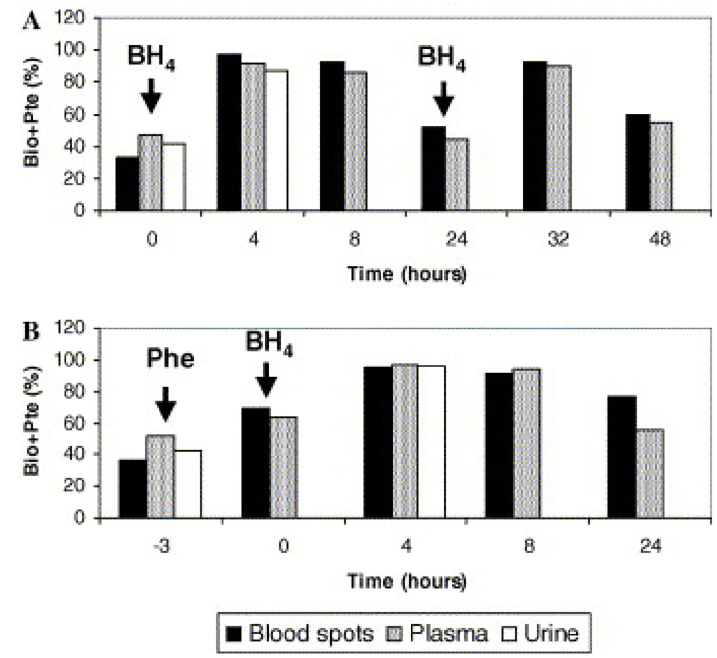

Fig. 5. Percentage of biopterin plus pterin (100 × (Bio + Pte)/(Neo + Bio + Pte)) in blood spots, plasma, and urine of two patients with HPA loaded with (A) 2 × 20mg/kg of BH_4 at T_0 and T_{24} hours (extended loading test); and (B) 100 mg/kg phenylalanine at T_{-3} and 20 mg/kg BH_4 at T_0 (combined loading test).

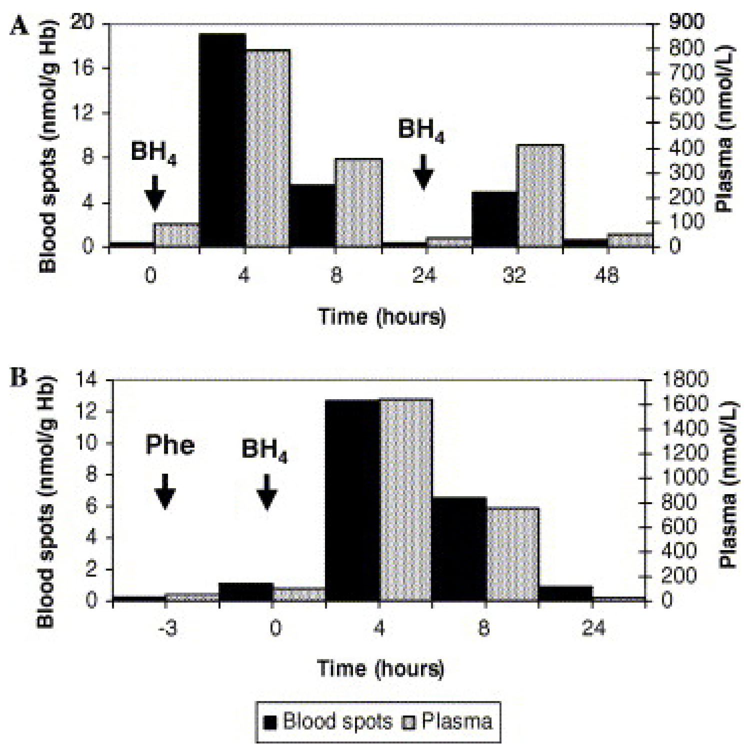

Fig. 6. Concentrations of biopterin plus pterin (Bio + Pte) in blood spots and plasma of two patients with HPA loaded with (A) 2 × 20 mg/kg of BH_4 at T_0 and T_{24} hours (extended loading test); and (B) 100 mg/kg phenylalanine at T_{-3} and 20 mg/kg BH_4 at T_0 (combined loading test).

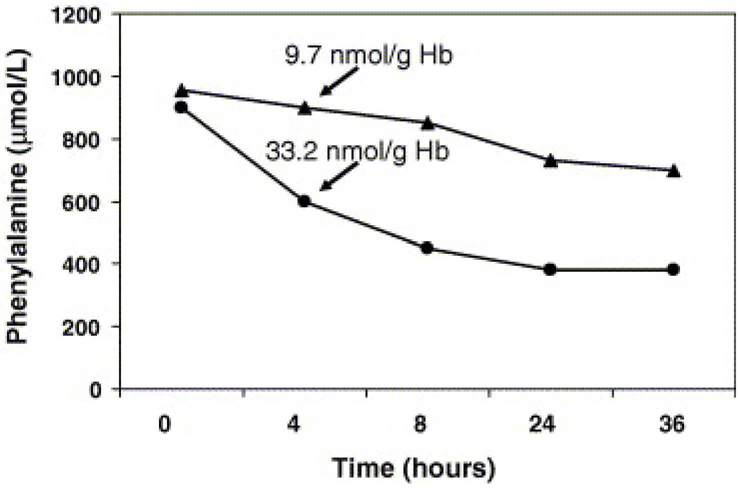

Fig. 7. Effect of oral administration of BH_4 (20 mg/kg) on blood phenylalanine levels in a patient with BH_4-responsive PAH genotype (A403V/S411X) on two separate loading tests. During the first test (slow response) T_4 blood spot BH_4 levels were 6.3 nmol/g Hb while during the second test (good response) BH_4 levels were ~3 times higher (33.2 nmol/g Hb).

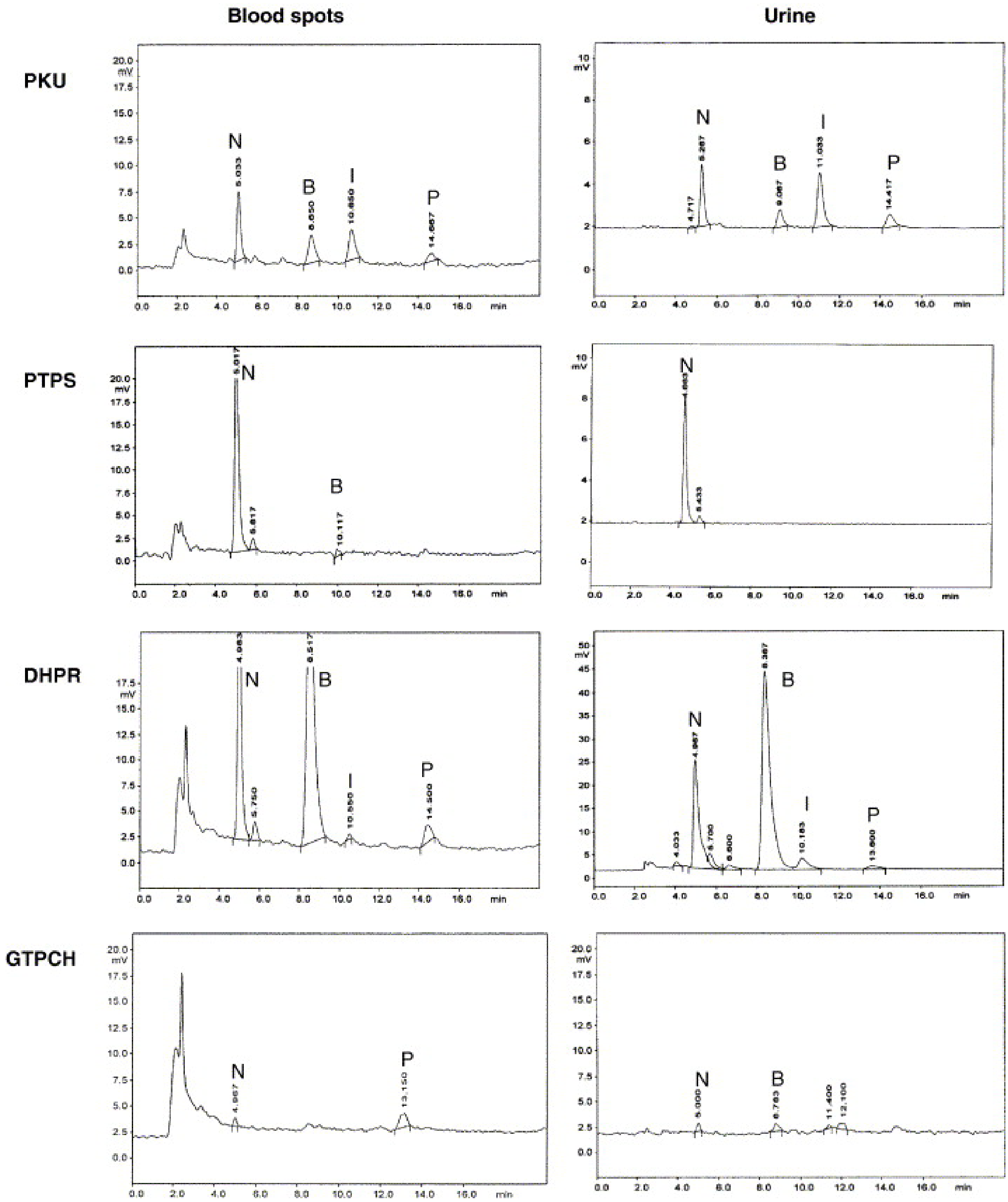

Fig. 8. HPLC profiles of pterins in blood spots and urine from patients with PKU and PTPS, DHPR, and GTPCH deficiencies. N: neopterin; B: biopterin; I: isoxanthopterin; P: pterin.

Discussion

Dried blood spots on filter paper (Guthrie cards) were introduced in the early 1960s for the newborn screening of few common and treatable inherited metabolic diseases, including PKU (Guthrie and Susi 1963). With the introduction of TMS, a number of new tests were developed for blood spots and blood collection on filter paper became a practical alternative for measurement of metabolites such as amino acids and acylcarnitines in serum, plasma, or even urine. A minimal sample volume is required and samples can be transported at room temperature in an envelope. In addition to measurement of phenylalanine and tyrosine, blood spots are used for measurement of DHPR activity in patients with HPA (Arai et al. 1982). These are routine tests for the screening of disorders in BH_4 metabolism and diagnosis is completed with the analysis of BH_4 metabolites in urine. Dried blood spots on Guthrie cards were also used for simultaneous measurement of total biopterin and DHPR activity. It used the *Crithidia fasciculata* bioassay, which may not detect biopterin levels in patients with partial or peripheral defects and was never tested for other BH_4 disorders (Leeming et al. 1984).

Depending on the profile of neopterin and biopterin in urine, enzyme defects in BH_4 metabolism can be localized. However, urine samples need to be oxidized or sent frozen on dry ice. We developed a method that allows measuring amino acids, DHPR activity, and pterins from a single Guthrie card (eight 6 mm diameter spots).

One of the main problems with the analysis of pterins in body fluids is their sensitivity to oxygen and light. Particularly, BH_4 is readily oxidized and degraded to biopterin and pterin, and depending where the degradation takes place, pterin is further oxidized to isoxanthopterin and xanthopterin by the xanthine dehydrogenase (Niederwieser et al. 1986). BH_4 is present in blood, CSF, and urine mainly in tetrahydro form (Fukushima and Nixon 1980) and in the circulating blood it is probably bound to albumin. In solutions, BH_4 is stable at acidic pH while at basic pH the side chain of BH_4 is split off, producing a pterin ring (Fukushima and Nixon 1980). We have previously shown that addition of ascorbic acid and DTE to plasma samples prevents BH_4 from both oxidation and degradation to pterin (Fiege et al. 2004). Using differential oxidation of plasma samples with iodine at acidic and basic pH, we were able to show that about 80% of total biopterin is present as BH_4. In dried blood spots on filter paper, BH_4 is already fully oxidized to biopterin and partially degraded to pterin. While the percentage of pterin is rather low in patients who are not on BH_4, it can be up to 50% after administration of BH_4. Once oxidized, pterins are stable in blood spots for up to 16 days at room temperature, which is the time estimated to be sufficient to send samples to the laboratory. Over longer period of time (up to 18 months), neopterin (102%) and biopterin (105%) are more stable than pterin (67%). There was a positive correlation between plasma and blood spots for biopterin + pterin ($r = 0.883$) and neopterin ($r = 0.842$) and similar correlation was found between urine and blood spots ($r = 0.852$ for biopterin + pterin and $r = 0.874$ for neopterin).

Elution and extraction of neopterin, biopterin, and pterin was optimized with respect to the eluent and time. Pterins are generally well soluble in hydrochloric acid and at the acidic pH and when stored in the dark, they are stable over a longer period of time. Addition of organic solvent, as used for the extraction of amino acids or acylcarnitines, did not improve extraction of pterins from dried blood spots. Recovery was calculated to be 63–69%. Concentrations of pterins in blood spots were expressed per haemoglobin content because of a relative high amount of neopterin and biopterin in red blood cells. We have previously shown that concentrations of biopterin are about two times higher in red blood cells than in plasma and this ratio is even higher for neopterin (eight times higher in red blood cells) (Ponzone et al. 1993).

Reproducibility is similar to those found for plasma pterins with a relative high run-to-run imprecision (24.0–52.9%) for the low-value sample, while the high-value run-to-run imprecision (14.3–17.0%) and both low (5.5–12.2%) and high-value (3.34–13.1%) within-run imprecision fulfil all diagnostic criteria.

Blood spots biopterin + pterin were used to monitor BH_4 pharmacokinetics in healthy controls and patients with HPA. Preliminary pharmacokinetic of BH_4 was already done in healthy volunteers by monitoring biopterin concentrations in plasma following oral administration of BH_4 (Fiege et al. 2004). As already mentioned, about 80% of total biopterin was found as BH_4 when analyzed immediately in antioxidant pre-treated plasma and without antioxidants no BH_4 was detected. Maximal BH_4 concentrations were found 1–4 h after BH_4 administration and the elimination half-life time was estimated to be 3.3–5.1 h (Fiege et al. 2004). We investigated blood spot pterins during the course of loading test with BH_4 in 53 patients with HPA (to be published elsewhere). Two loading tests are presented in this paper and blood spot biopterin + pterin were compared with plasma biopterin (Fig. 6). In the first loading test, two dosages of BH_4 (2 × 20 mg/kg) were administered and biopterin + pterin was monitored up to 48 h after administration. As expected, maximal levels were found at T_4 and T_{32} hours and blood spots and plasma levels were comparable. Similarly, in the second loading test (combined 100 mg/kg phenylalanine and 20 mg/kg BH_4 challenge) biopterin + pterin slightly increased in blood spots and plasma 3 h after phenylalanine administration and peaked 4 h after BH_4 administration. Thus, measurement of pterins in blood spots seems to be useful for monitoring BH_4 pharmacokinetics. This was also demonstrated in a patient who was based on the genotype (A403V/S411X) predicted to be a BH_4-responder. As shown in Fig. 7, there was only slow decrease in blood phenylalanine concentration after administration of BH_4 (20 mg/kg). At that time, blood spot biopterin + pterin levels were 9.7 nmol/g Hb. The test was repeated several weeks later and the patient was found to be a good BH_4-responder with blood spot biopterin + pterin levels of 33.2. Obviously, intra-individual variation in BH_4 absorption may contribute to BH_4 blood levels as documented by monitoring blood spot biopterin + pterin.

In a pilot study of more than 70 patients with HPA, we measured blood spot pterins before the BH_4 loading test and compared results with the standard screening in urine. Four patients were detected to be BH_4-deficient; one patient with the GTPCH deficiency, two with PTPS deficiency, and one with DHPR deficiency. The profile of pterins in blood spots was identical with that found in urine collected at the same time (Fig. 8). Although, the number of patients tested is rather small and no patients with PCD deficiency were detected so far, preliminary results suggest that blood spots on filter paper may be a practical alternative option for the differential diagnosis of common forms of BH_4 deficiency. Eight 6 mm spots are sufficient for the analysis of pterins (four spots), DHPR activity (two spots), and amino acids (two spots).

Acknowledgements

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Chapter 3

PHARMACOKINETICS OF ORALLY ADMINISTERED TETRAHYDROBIOPTERIN IN PATIENTS WITH PHENYLALANINE HYDROXYLASE DEFICIENCY

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Summary

The oral loading test with tetrahydrobiopterin (BH_4) is used to discriminate between variants of hyperphenylalaninaemia and to detect BH_4-responsive patients. The outcome of the loading test depends on the genotype, dosage of BH_4, and BH_4 pharmacokinetics. A total of 71 patients with hyperphenylalaninaemia (mild to classic) were challenged with BH_4 (20 mg/kg) according to different protocols (1 × 20 mg or 2 × 20 mg) and blood BH_4 concentrations were measured in dried blood spots at different time points (T_0, T_2, T_4, T_8, T_{12}, T_{24}, T_{32} and $T_{48\ h}$). Maximal BH_4 concentrations (median 22.69 nmol/g Hb) were measured 4 h after BH_4 administration in 63 out of 71 patients. Eight patients presented with maximal BH_4 concentrations ~44% higher at 8 h than at 4 h. After 24 h, BH_4 blood concentrations dropped to 11% of maximal values. This profile was similar using different protocols. The following pharmacokinetic parameters were calculated for BH_4 in blood: t_{max} = 4 h, AUC ($T_{0–32}$) = 370 nmol × h/g Hb, and $t_{1/2}$ for absorption (1.1 h), distribution (2.5 h), and elimination (46.0 h) phases. Maximal BH_4 blood concentrations were not significantly lower in non-responders and there was no correlation between blood concentrations and responsiveness. Of mild PKU patients, 97% responded to BH_4 administration, while one was found to be a non-responder. Only 10/19 patients (53%) with Phe concentrations of 600–1200 µmol/L responded to BH_4 administration and of the patients with the severe classical phenotype (blood Phe > 1200 µmol/L) only 4 out of 17 patient responded. An additional 36 patients with mild hyperphenylalaninaemia (HPA) who underwent the combined loading test with Phe+BH_4 were all responders. Slow responders and non-responders were found in all groups of HPA.

Abbreviations

AUC area under the curve

BH_4 tetrahydrobiopterin

HPA hyperphenylalaninaemia

PAH phenylalanine hydroxylase

PKU phenylketonuria

$t_{1/2}$ half-life

Introduction

The loading test with tetrahydrobiopterin (BH_4) is an essential and integral part of the differential diagnosis for hyperphenylalaninaemia (HPA) (Blau et al. 2001). This test discriminates between BH_4 responders and non-responders and is particularly important for detection of patients with BH_4-responsive phenylalanine hydroxylase (PAH; EC 1.14.16.1) deficiency (Blau and Erlandsen 2004). In BH_4 responders, blood phenylalanine (Phe) declines 4–24 h after BH_4 administration (20 mg/kg body weight), with almost complete normalization after 4–8 h in patients with BH_4 deficiency. BH_4-responsive PAH patients were initially defined as showing a decrease of blood Phe concentrations of 30% after 8 h and 30–50% after 24 h (Bernegger and Blau 2002) and most of them belong to the group of mild HPA and mild and moderate phenylketonuria (PKU; OMIM 262600) (Desviat et al. 2004; Fiori et al. 2005; Kure et al. 1999; Lässker et al. 2002; Lindner et al. 2003; Lücke et al. 2003; Matalon et al. 2004; Mitchell et al. 2005; Muntau et al. 2002; Pérez-Dueñas et al. 2004; Spaapen et al. 2001; Steinfeld et al. 2003; Trefz and Blau 2003). Sensitivity of the test was further improved by multiple administrations of BH_4 and by longer observation time (Fiege et al. 2005; Shintaku et al. 2004). Efficiency and interpretation of the loading test depends on several factors such as amount of administered BH_4, severity of HPA, dietary management, and genotype. The pharmacokinetics of BH_4 was suggested as an additional factor affecting the loading test; however, this was never investigated in patients with PKU (Fiege et al. 2004).

The aim of this study was to estimate basic kinetic parameters for BH_4 in blood after administration of BH_4 and following the combined Phe+BH_4 loading test, and to correlate the BH_4 concentrations in blood with the outcome of the test.

Materials and Methods

Patients

A total of 71 HPA patients in whom BH_4 deficiency had been excluded were loaded with a single dose of BH_4 (20 mg/kg); 35 of them presented with basal blood Phe concentrations <600 µmol/L, 19 with Phe concentrations of 600–1200 µmol/L, and 17 with Phe concentrations >1200 µmol/L. In 11 patients with blood Phe concentrations of 278–1575 µmol/L the standard test was extended by the administration of another 20 mg/kg BH_4 after 24 h. An additional 36 patients with basal Phe concentrations of 384–739 µmol/L underwent a combined Phe (100 mg/kg) and BH_4 (20 mg/kg) loading test.

Mutation analysis was done in only a few patients and was not included in this study. The study was performed after a formal consensus of patients or their parents and in accordance with the Helsinki recommendations 1989.

Loading test.

The single BH_4 test, the extended test, and the combined Phe+BH_4 loading test were performed as described previously using the 6R-BH_4 (Schircks Laboratories, Jona, Switzerland) (Blau and Erlandsen 2004; Fiege et al. 2005). In patients loaded with a single dose of BH_4 (20 mg/kg), blood was collected at times T_0, T_4, T_8 and $T_{24\,h}$. In 45 of them additional blood collections were done at T_2, T_{12} and $T_{48\,h}$. In patients loaded with 2 × 20 mg/kg BH_4, blood was collected at T_0, T_4, T_8, T_{24}, T_{32} and $T_{48\,h}$, and in patients loaded with Phe+BH_4 the following blood collections were performed: T_{-3} (Phe administration), T_0 (BH_4 administration), T_4, T_8 and $T_{24\,h}$.

The following criteria were used to define BH_4-responsiveness over 24 h (Fiege et al. 2005): ’rapid responder’, reduction of blood Phe by ≥30% at T_8, and ≥50% at T_{24}; ’moderate responder’, reduction of blood Phe by ≥20% at T_8, and ≥30% at T_{24}; ’slow responder’, reduction of blood Phe by <20% at T_8, and ≥20% at T_{24} (Fig. 1).

BH_4 and Phe in blood

BH_4 was measured in dried blood spots according to the method of Zurflüh and colleagues (Zurflüh et al. 2005) and calculated as the sum of total biopterin and pterin (nmol BH_4/g Hb). The following protocol was used: For each measurement, four blood circles (Ø6 mm) were cut out and pterins were extracted with 250 µL of 20 mmol/L HCl and placed in an ultrasonic bath for 30 s. Pterins were extracted by mixing the filter spot solution for 10 min at room temperature. The extract was centrifuged at 1800×*g* for 5 min at room temperature. Clear supernatatant (60 µL) was used for analysis of haemoglobin on the haematology analyser Sysmex KX-21N (Sysmex Corporation, Japan). The remaining supernatant was ultrafiltered on Ultrafree (NMWL 10000; Millipore) at 5000×*g* for 15 min. Pterins were analysed in clear filtrate by HPLC and fluorescence detection without prior oxidation (Zurflüh et al. 2005).

Phe was measured using standard ion-exchange chromatography of amino acids or tandem-mass spectrometry.

Statistical analyses

WinSTAT for Excel (v. 2003.1) was used for descriptive statistics and for regression analysis. Pharmacokinetic parameters were calculated using the PK Solutions software, v. 2.0 (Summit Research Services, Montrose, CO).

Results

BH_4 kinetics in blood

In 63 out of 71 patients with HPA loaded with 20 mg/kg BH_4, blood BH_4 concentrations reached highest values after 4 h (median = 22.65 nmol/g Hb; 5^{th}–95^{th} percentiles = 10.07–49.97 nmol/g Hb). Four hours later (T_8) BH_4 concentrations decreased by 42% (median = 13.61 nmol/g Hb; 5th–95^{th} percentiles = 5.25–31.63 nmol/g Hb) and after 24 h BH_4 concentrations were only 11% of maximal values (median = 2.29 nmol/g Hb; 5th–95th percentiles = 0.67–5.35 nmol/g Hb). In 8 out of 71 patients, blood BH_4 concentrations were ~44 % higher at 8 h than values at 4 h after administration and the highest single BH_4 value was 96.39 nmol/g Hb at $T_{4\,h}$.

In 45 of the above patients the blood BH_4 profile was investigated over 32 h after BH_4 administration (20 mg/kg) with additional time points at T_2, T_{12} and $T_{32\,h}$ (Fig. 2). Two hours after BH_4 administration (median = 16.30 nmol/g Hb; 5^{th}–95^{th} percentiles = 6.84–28.44 nmol/g Hb) blood concentrations reached about 70% of the maximal concentrations found after 4 h. At 12 h (median = 6.15 nmol/g Hb; 5^{th}–95^{th} percentiles = 2.90–12.38 nmol/g Hb) and 32 h (median = 2.03 nmol/g Hb; 5^{th}–95^{th} percentiles = 0.51–5.47 nmol/g Hb) BH_4 blood concentrations were lower than those at 8 h (47% and 15%, respectively).

In 11 patients the protocol was extended to a second administration of BH_4 (20 mg/kg) after 24 h (Fig. 3). Blood was collected 8 (T_{32}) and 24 (T_{48}) hours after the second administration and compared with T_8 concentrations for BH_4 in blood (median = 15.37 nmol/g Hb; 5^{th}–95^{th} percentiles = 7.05–54.02 nmol/g Hb); T_{32} values were 17% lower (median = 12.80 nmol/g Hb; 5^{th}–95^{th} percentiles = 7.41–38.54 nmol/g Hb). There was no significant difference in BH_4 concentrations at T_{24} and $T_{48\,h}$.

Thirty-six patients with blood Phe concentrations of <336 μmol/L were loaded first with Phe (100 mg/kg) and three hours later with BH_4 (20 mg/kg), and blood samples were collected before Phe administration (T_{-3}), before BH_4 administration (i.e. 3 h after Phe loading; T_0), and 4, 8 and 24 h after BH_4 administration ($T_{4\text{-}24}$) (Fig. 4). BH_4 concentrations in blood increased in 26/36 patients after Phe administration by 111% (BH_4 at T_{-3}, median = 0.30 nmol/g Hb; 5^{th}–95^{th} percentiles = 0.12–2.77 nmol/g Hb; BH_4 at T_0, median = 0.52 nmol/g Hb; 5^{th}–95^{th} percentiles = 0.16–4.43 nmol/g Hb). The following profile of BH_4 kinetics was similar as the one described for a single BH_4 administration, with maximal blood concentrations at T_4 (median = 22.01 nmol/g Hb; 5^{th}–95^{th} percentiles = 10.40–46.58 nmol/g Hb), 25% lower concentrations at T_8 (median = 16.46 nmol/g Hb; 5^{th}–95^{th} percentiles = 66–33.49 nmol/g Hb), and 90% lower concentrations at T_{24} (median = 2.09 nmol/g Hb; 5^{th}–95^{th} percentiles = 0.55–6.84 nmol/g Hb). In 6 subjects maximal BH_4 concentrations were reached after 8 h.

Based on data obtained over 32 h after a single BH_4 administration (20 mg/kg) in 45 patients with HPA, basic pharmacokinetic parameters were calculated: t_{max} was 4 h, AUC ($T_{0\text{-}32}$) was 370 nmol × h/g Hb, and $t_{1/2}$ for absorption, distribution, and elimination phases was 1.1, 2.5, and 46.0 h, respectively.

Responsiveness to BH_4

Table 1 summarizes the results of the loading test with 20 mg/kg BH_4 in patients with HPA. Responsiveness was calculated according to criteria defined in Fig. 1. 34/35 patients (97%) with basal blood Phe concentrations <600 μmol/L responded to BH_4 administration, one patient was found to be a non-responder. Only 10/19 patients (53%) with basal blood Phe concentrations of 600–1200 μmol/L responded to BH_4 administration, and of the patients with the severe classical phenotype (blood Phe >1200 μmol/L) only four patients responded (24%), and two of them were slow responders (Fig. 5). The lower the Phe at T_0 the higher the probability that a patient will respond to BH_4. Two patients could not be assigned to the above-mentioned criteria; one would have been positioned somewhere between moderate and slow responder and the other was at the level of a rapid responder after 8 h but dropped at 24 h below the level of a slow responder.

All 36 patients who underwent the combined loading test with Phe+BH_4 were classified as responders (data not shown).

In order to demonstrate whether absorption of BH_4 may affect the outcome of the loading test, maximal blood BH_4 concentrations were compared with responsiveness to BH_4, but no correlation was found (Fig. 6). Highest maximal blood BH_4 concentrations were found in two rapid responders.

Figures and Tables

Table 1.
Summary of BH_4 loading tests (20 mg/kg) in 71 patients with PAH deficiency

		Initial blood Phe concentrations		
	n	<600 µmol/L (*n* = 35)	600–1200 µmol/L (*n* = 19)	>1200 µmol/L (*n* = 17)
Responder (total)	48	34	10	4
Rapid responder	34	26	6	2
Moderate responder	10	7	3	0
Slow responder	4	1	1	2
Non-responder	21	1	8	12
Not defined	2	0	1	1

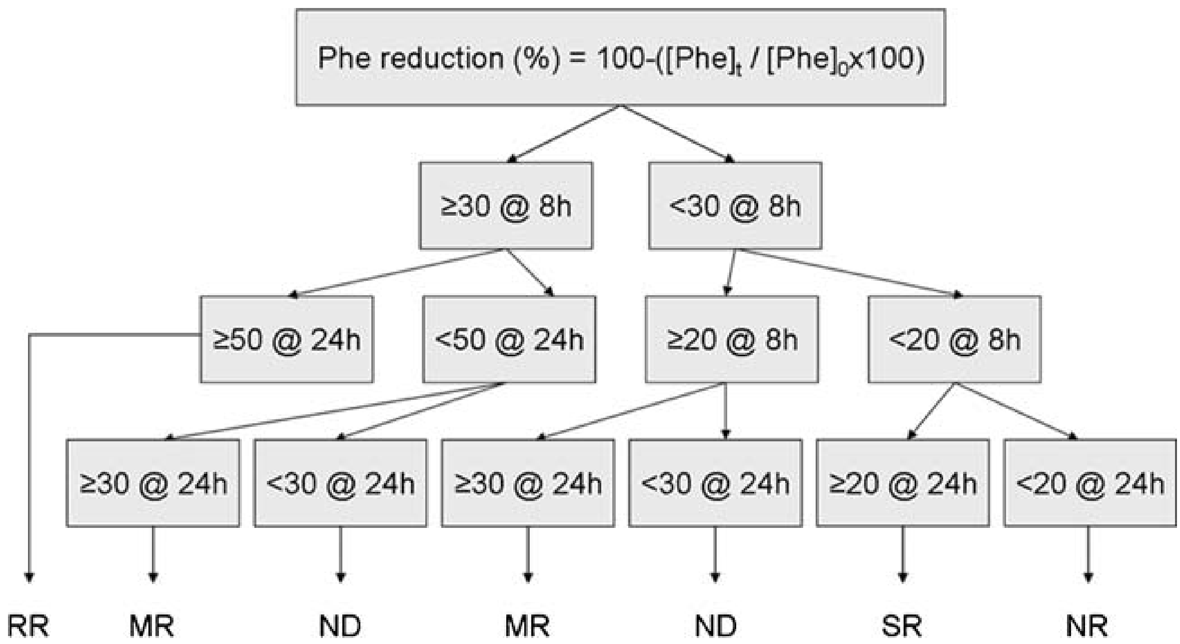

Fig. 1. Definition of the BH_4 responsiveness after oral administration of BH_4 (20 mg/kg). RR: rapid responder; MR: moderate responder; SR: slow responder; NR: non-responder; ND not defined

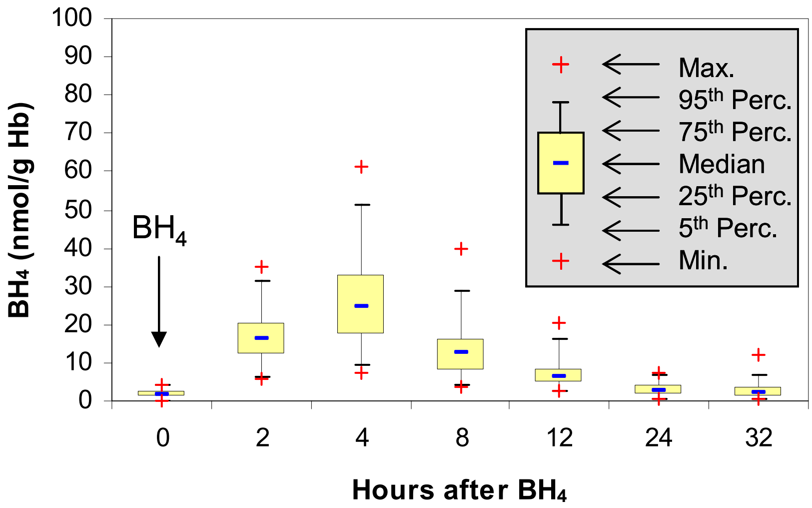

Fig. 2. BH_4 concentrations in blood after oral administration of BH_4 (20 mg/kg) in 47 patients with HPA. — Median, □ 25^{th}–75^{th} percentiles; ⊥ 5^{th} percentile; ┬ 95^{th} percentile; + min/max

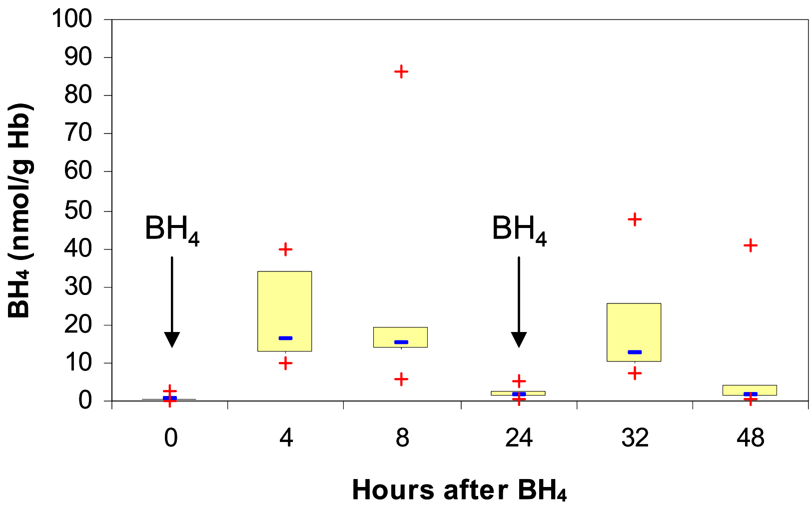

Fig. 3. BH_4 concentrations in blood after oral administration of BH_4 (2 × 20 mg/kg) in 11 patients with HPA. Second dosage of BH_4 was administered 24 h after the first application. — Median, □ 25^{th}–75^{th} percentiles; ⊥ 5^{th} percentile; ┬ 95^{th} percentile; + min/max

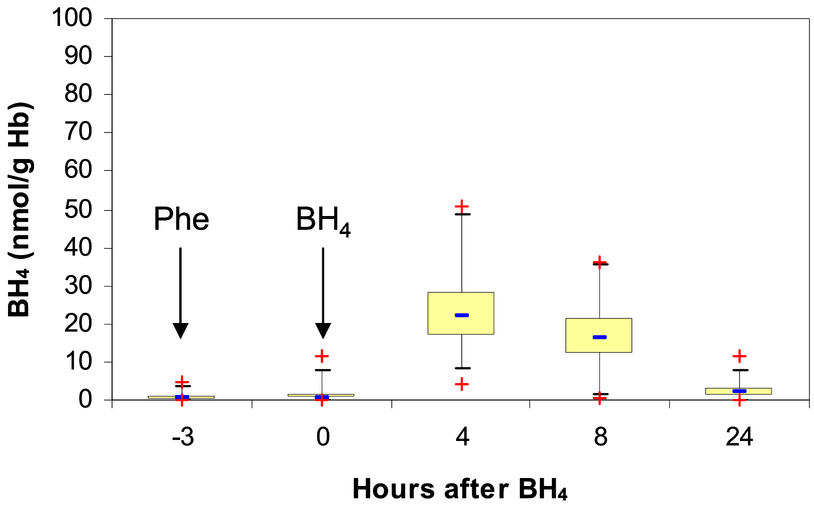

Fig. 4. BH_4 concentrations in blood after oral administration of Phe (100 mg/kg) and BH_4 (20 mg/kg) in 36 patients with mild HPA. BH_4 was administered 3 h after Phe application. — Median, □ 25th–75th percentiles; ⊥ 5th percentile; ┬ 95th percentile; + min/max

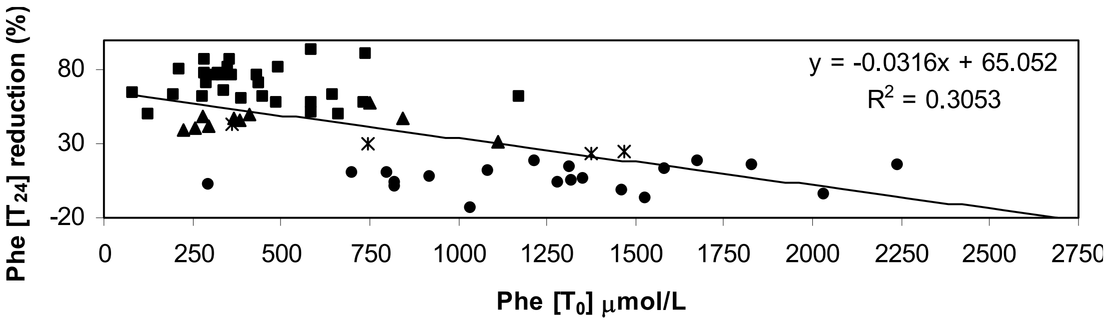

Fig. 5. Correlation between Phe decline in blood 24 h after administration of BH_4 (20 mg/kg) and basal Phe levels in 71 patients with HPA. ■ Rapid responder; ▲ moderate Responder, * slow responder; ● non-responder

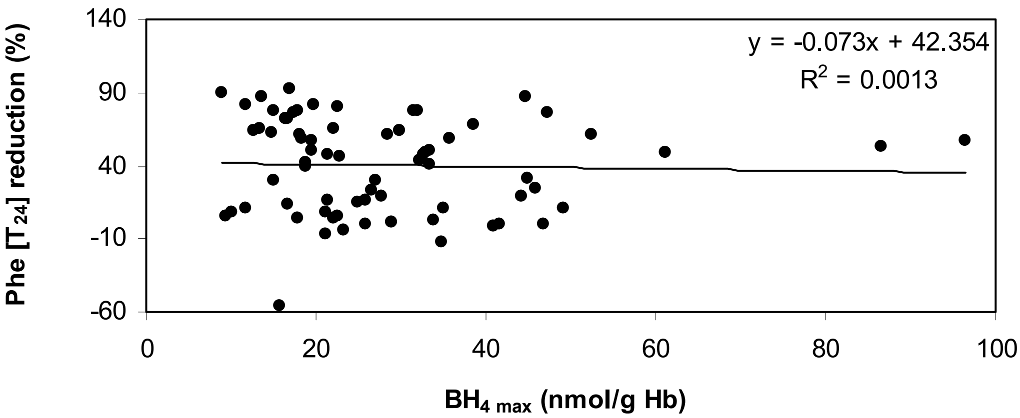

Fig. 6. Correlation between Phe decline in blood 24 h after administration of BH_4 (20 mg/kg) and maximal BH_4 levels in 71 patients with HPA

Discussion

Extensive pharmacokinetic studies of BH_4 have been performed in animal models (Hayashi et al. 1992), but only a few parameters are known from studies in humans (Følling 2006). Some pharmacokinetic parameters are known from oral administration of BH_4 tablets to healthy adult human volunteers and might provide details on pharmacological response to BH_4 therapy (Fiege et al. 2004). Plasma concentrations of BH_4 and total biopterin were assessed after oral administration of 6R-BH_4 at different doses to different healthy subjects and preliminary pharmacokinetic parameters have been determined (Fiege et al. 2004). The plasma profile of total biopterin after oral administration exhibited first-order kinetics, showing a fast absorption phase (T_0–T_4), a rapid decline (T_4–T_{10}) corresponding to the absorption and distribution phase, followed by a slower decline in the final elimination phase (T_{10}–$T_{33\,h}$). Total biopterin concentrations in plasma have been studied after administration of different doses (10 and 20 mg/kg) to one healthy adult subject (Fiege et al. 2004). Maximal plasma concentrations in this subject were reached 4 h after the 10 mg/kg dose and 3 h after the 20 mg/kg dose, at concentrations of 258.7 and 441.7 nmol/L, respectively. The $AUC_{0\text{-}10}$ after administration of 20 mg/kg was 1.6 times higher than the AUC after the 10 mg/kg dosage (3046 vs. 1958 nmol h/L). Based on these data, the elimination kinetics seem to be only slightly faster at higher plasma concentrations (t_{max} = 4.2 h vs. 5.1 h) (Fiege et al. 2004).

Very little is known about BH_4 pharmacokinetics in patients with HPA. Shintaku and colleagues (Shintaku et al. 2005) reported plasma biopterin concentrations in two patients with HPA who underwent a single BH_4 loading test (10 mg/kg) at different ages. In both patients plasma biopterin concentrations were ~100% higher at an early age (<1 month) compared with concentrations measured at the age of 2 months. Also, biopterin concentrations peaked at 4 h at the age of 1 month, compared with maximal concentrations at 2 h at the age of 2 months. The authors suggested that BH_4-responsiveness in the same individual or the same genotype may correlate with biopterin concentration, but in different genotypes this might not be the case (Shintaku et al. 2005).

We were not able to see any statistical difference between different age groups in our patients (data not shown). Similarly to what was described for healthy controls (Fiege et al. 2004), blood BH_4 peaked at 4 h in 90% of patients with HPA. This profile was consistent regardless of whether patients were loaded with one or two BH_4 doses or after Phe administration (Figs. 2–4). Two hours after BH_4 administration, blood concentrations were about 70% of the maximal BH_4

concentrations, indicating a very fast absorption phase ($t_{1/2}$ = 1.1 h). In our patients, oral administration of BH_4 resulted in a fast distribution phase ($t_{1/2}$ = 2.5 h), followed by a slow elimination phase ($t_{1/2}$ = 46.0 h). Thus, although a single BH_4 administration may be sufficient for the interpretation of the loading test, additional dosages can potentate the effect and increase the sensitivity (Fiege et al. 2005; Shintaku et al. 2004).

Data from the combined Phe+BH_4 loading test show that administration of Phe (100 mg/kg) almost doubled blood BH_4 concentrations after 3 h in 72% of patients (Fig. 4). This is consistent with previous findings that biopterin concentrations in urine or plasma correlate with blood phenylalanine concentrations (Dhondt and Farriaux 1982; Ponzone et al. 1993) but have no consequences on the outcome of the loading test. Phenylalanine administration does not influence BH_4 concentrations upon oral BH_4 administration. As expected, all patients in this group were classified as responders and one should question how useful this test is. Factors such as spontaneous Phe elimination (Desviat et al. 2004) or daily fluctuations (Leuzzi et al. 2006) may influence the interpretation, and from our experience the combined loading test is not recommended. It can be only used in patients who are already on a strict low-phenylalanine diet with normalized blood phenylalanine concentrations.

One of the main goals of this study was to evaluate the effect of blood BH_4 concentrations on the outcome of the loading tests. Recently we described a single case with HPA (BH_4-responsive genotype) showing intra-individual variations in BH_4 absorption on two occasions, which resulted in different BH_4 blood concentrations and influenced the responsiveness (Zurflüh et al. 2005). We were not able to repeat the loading test in non-responders in this study, but maximal BH_4 blood concentrations were not significantly lower in this group of patients. Indeed, maximal BH_4 blood concentrations were only 3% lower in non-responders than in all responders, and slow responders had 7% and 42% higher concentrations than moderate or rapid responders, respectively (data not shown).

With regard to the responsiveness to BH_4, our data confirm previous observations that rapid responders belong mainly to the groups of mild HPA and mild PKU, and that patients with classical PKU show either only a slow response or none at all (Fig. 5). Nevertheless, slow responders and non-responders were found in all groups of HPA.

Acknowledgements

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Chapter **4**

MOLECULAR GENETICS OF TETRAHYDROBIOPTERIN-RESPONSIVE PHENYLALANINE HYDROXYLASE DEFICIENCY

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Abstract

Mutations in the phenylalanine hydroxylase (PAH) gene result in phenylketonuria (PKU). Tetrahydrobiopterin (BH_4)-responsive hyperphenylalaninemia has been recently described as a variant of PAH deficiency caused by specific mutations in the *PAH* gene. It has been suggested that BH_4-responsiveness may be predicted from the corresponding genotypes. Data from BH_4 loading tests indicated an incidence of BH_4-responsiveness of >40% in the general PKU population and >80% in mild PKU patients. The current project entailed genotype analysis of 315 BH_4-responsive patients tabulated in the BIOPKUdb database and comparison with the data from the PAHdb locus-specific knowledgebase, as well as with previously published *PAH* mutations for several European countries, Northern China, and South Korea. We identified 60 mutations, presenting with a substantial residual PAH activity (~47%), presumed to be associated with BH_4-responsiveness. More than 89% of patients are found to be compound heterozygotes. The three most common mutations found in >5% of BH_4-responsive patients are p.A403V, p.R261Q, and p.Y414C. Using the Hardy-Weinberg formula the predicted average frequency of BH_4-responsiveness in European populations was calculated to be 55% (range 17-79%, lowest in Baltic countries and Poland and highest in Spain), 57% in Northern China, and 55% for South Korea. The genotype-predicted prevalence of BH_4-responsiveness was higher than prevalence data obtained from BH_4 loading tests. Inconsistent results were observed for mutations p.L48S, p.I65T, p.R158Q, p.R261Q, and p.Y414C. Our data suggest that BH_4-responsiveness may be more common than assumed and to some extent may be predicted or excluded from the patient's genotype.

Introduction

Phenylketonuria (PKU; OMIM# 261600) is an autosomal recessive genetic disorders caused by a deficiency of the hepatic phenylalanine-4-hydroxylase (PAH; EC 1.14.16.1) (Scriver and Kaufman 2001). PAH is a mixed function oxidase that catalyzes hydroxylation of phenylalanine (Phe) to tyrosine, the rate-limiting step in phenylalanine catabolism. The reaction is dependent on BH_4 as cofactor, molecular oxygen, and iron. PAH deficiency causes hyperphenylalaninaemia (HPA) of variable degree. A distinction is made between PKU which requires a phenylalanine-restricted diet, and mild hyperphenylalaninaemia (MHP), a variant that does not require treatment. Depending on the phenylalanine tolerance, PKU may be divided into mild PKU, moderate PKU, and classical PKU (Guldberg et al. 1998); some centers only distinguish between mild and classical PKU. The *PAH*-mutation genotype is the main determinant of the metabolic phenotype. Recently Kure et al. (Kure et al. 1999) described patients who responded to oral administration of BH_4 by lowering their blood phenylalanine levels and subsequently several groups documented that BH_4 can be successfully used for the long-term treatment of HPA patients as an alternative to a dietary treatment (Bélanger-Quintana et al. 2005; Cerone et al. 2004; Hennermann et al. 2005; Lambruschini et al. 2005; Muntau et al. 2002; Shintaku et al. 2004; Steinfeld et al. 2004; Trefz et al. 2001; 2005). It has been estimated that, depending on the criteria selected, more than 30% of all HPA patients respond to BH_4 (20 mg/kg) (Bernegger and Blau 2002) and that the main target for BH_4 administration are patients with mild to moderate PKU (Fiege and Blau 2007).

Molecular mechanisms responsible for BH_4-responsiveness in patients with PKU are multifactorial (Aguado et al. 2006; Blau and Erlandsen 2004; Erlandsen et al. 2003, 2004; Kure et al. 2004; Pey et al. 2004; Steinfeld et al. 2003; Thöny et al. 2004) and depend on mutations in the *PAH* gene. Chaperon-like activity of BH_4 and stabilization of PAH protein and a considerable residual activity as well as an increase in intracellular BH_4 concentrations required for the full enzyme activity are some of the proposed mechanisms. Mutations so far described in patients with BH_4-responsive HPA are tabulated in the BIOPKU database (BIOPKUdb) and many of them have been shown to be associated with a significant residual PAH activity, when recombinantly expressed in different cell systems.

In order to estimate the frequency of BH_4-responsiveness in different populations based on genotype information, we calculated the allele and genotype frequencies for the BIOPKUdb and compared them with data published for different countries, including the PAHdb locus knowledgebase (Scriver et al. 2003).

Subjects and Methods

Source of data

Data from 315 patients who responded to the BH_4 loading test (10-20 mg/kg) by lowering their blood phenylalanine levels by >30% after 8-24 hours, were tabulated in the International Database of Patients and Mutations Causing BH_4-Responsive HPA/PKU (BIOPKUdb, http://www.bh4.org/BH4DatabasesBiopku.asp, programmed in Access 2003, Microsoft, Redmond, WA). The minimal requirement for inclusion in the database was a 30% blood phenylalanine reduction 8 hour after administration of 10 mg/kg BH_4. It is assumed that patients loaded with 20 mg/kg BH_4 over 24 hours would have the same or even better responsiveness. Therefore, data were not stratified in separate groups. Dietary intake of phenylalanine and initial blood phenylalanine levels were different, depending on the actual phenotype. BIOPKUdb includes detailed information on genotype (mutation name, systemic name, location in gene, PAH domain, residual activity, and HPA type), BH_4 loading test data (amount of BH_4 used and percentage of responsiveness), and references.

Both protein and cDNA numbers follow standard recommendations with nucleotide +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, and the initiation codon denoted codon 1. Reference sequences: GenBank cDNA = NM_000277.1; protein = NP_000268.1.

PAHdb, http://www.pahdb.mcgill.ca, a locus-specific knowledgebase (Scriver et al. 2006) tabulates a total of 3137 alleles (regardless of BH_4-responsiveness) from countries all over the world. In 633 patients the genotype is defined (last access April 2007). In addition to molecular data, information about the source of alleles, polymorphic haplotype backgrounds, and effect of the allele on the enzyme activity is provided. Little information is provided about BH_4-responsiveness.

Allele frequencies of common PKU mutations in Europe were previously published (Zschocke and Hoffmann 1999); we included studies only if the mutation detection rate exceeded 80%. Full genotype data were available for 119 patients from Northern Ireland (60 different genotypes) (Zschocke et al. 1995) and 275 patients from Germany (165 different genotypes) (Zschocke and Hoffmann 1999). Information on BH_4-responsiveness was not available for those cohorts. In addition to European patients we also included published patients from Korea and China with fully characterized genotypes in our study. There were 58 patients with 48 different genotypes from Korea (the original study comprised 79 patients, but achieved only a mutation detection rate of 75%) (Lee et al. 2004). There were 155 patients with 97 different genotypes from China (the original study comprised 185 patients and achieved a mutation detection rate of 94 %) (Song et al. 2005).

Data limitation

Excluded from the study are patients with only one allele detected (13 patients) and those with two null alleles (no residual activity) (3 patients).

Criteria for BH_4-responsiveness

The criteria of BH_4-responsiveness in patients with PAH deficiency have been previously described (Blau and Erlandsen 2004); briefly, patients were classified as BH_4-responsive if they responded to the oral administration of BH_4 (10-20 mg/kg body weight) by lowering their blood

Phe levels by at least 30% within 8 to 24 hours. A mutation/an allele was regarded as associated with BH_4-responsiveness if it was present either in homozygous form or compound heterozygous associated with a known null mutation. Mutations were also regarded as null mutations when in vitro residual activity was below 10% and the second mutation in a compound heterozygous BH_4-responsive patient had a substantial residual PAH activity (>10%). In vitro enzymatic activities (mutant PAH as a percentage of wild-type PAH) are generally higher than those reported in liver biopsies (Waters et al. 1998).

Alleles that could not be classified using these criteria were labeled unclear.

Population genetics

In order to estimate the frequency of BH_4-responsiveness in different populations we regarded BH_4-responsive mutations as dominant in compound heterozygous patients. BH_4-responsiveness was expected in all patients with a BH_4-responsive mutation on at least one *PAH* gene copy. For populations without available genotypes, the expected frequency of genotypes associated with BH_4-responsiveness was estimated using the Hardy-Weinberg formula, as follows. We first calculated the combined allele frequencies for BH_4-responsive mutations and non-responsive mutations (including unclear alleles), respectively. Subsequently, squared combined frequency of non-responsive alleles was taken as the frequency of non-responsive genotypes, whilst the other genotypes were regarded as BH_4-responsive.

Results

BIOPKUdb

The BIOPKUdb comprises genotypes information from 315 BH_4-responsive patients. 115 different *PAH* gene mutations were found, 57% of which were present in more than one patient (Table 1). About 50% of the mutations described in the BIOPKUdb were previously expressed recombinantly in eukaryotic cell systems or in *E. coli*, and many were found to have substantial residual activity (>10%). The average residual PAH activity calculated for the 40 most common BH_4-responsive mutations was 46.8% (data not shown).

Using the criteria outlined 57 mutations were classified as BH_4-responsive (Figure 1), 20 were classified as non-responsive and 38 mutations were termed unclear. The most common BH_4-responsive mutations in the database are p.A403V (56 alleles), p.R261Q (49 alleles), p.Y414C (44 alleles), p.A300S and p.V245A (26 alleles each), p.L48S (24 alleles), p.E390G (23 alleles), and p.R241C (20 alleles) (Table 1). 32 out of 315 patients were homozygous, 102 had two different BH_4-responsive alleles, and 173 had one BH_4-responsive allele and one either non-responsive or unclear allele. Eight patients (2.5%) were compound heterozygous for two unclear alleles. The most common BH_4-responsive genotypes are listed in the Supplementary Table S1.

The mutations collected in the BIOPKUdb are spread all over the *PAH* gene (Figure 1). Only two out of 60 BH_4-responsive mutations were located in the cofactor binding domain CBR1 (p.V245A and p.R261Q). The overall distribution of the different mutations (responsive and non-responsive) tabulated in the BIOPKUdb is as follows: 64% are found in the catalytic domain, 14% are in the regulatory domain, 10% in the tetramerization domain, and 12% are intronic. In comparison to non-responsive mutations, responsive mutations are located with an increased percentage in the tetramerization (14% vs. 1%) and the regulatory domains (16% vs. 1%). Most of the responsive mutations were found in the catalytic domain (68%) (Table 2). There was a small number of splicing mutations that are BH_4-responsive, contrary to the usual experience that they represent null mutations. This phenomenon may be caused by residual normal splicing with low efficiency or the generation of novel transcripts with in-frame insertions or deletions compatible with residual enzyme function (Jennings, et al., 2000). Nevertheless, it remains to be seen whether BH_4 sensitivity associated with splicing mutations can be confirmed in other patients and how this effect is mediated by BH_4.

PAH database and BH_4-responsiveness

Based on the information in the BIOPKUdb, mutations tabulated in the PAHdb were assigned as BH_4-responsive, non-responsive, or unclear (see Materials and Methods for the definition) and predicted frequencies are calculated using the Hardy-Weinberg equation.

The 3173 mutations in the PAHdb were classified into responsive alleles (32%) and non-responsive alleles (68%, incl. unclear and mutations not found in the BIOPKUdb). For 633 patients, genotypes were defined and it was found that within this group of patients responsive alleles make up 36% of the mutations (Table 3). Study of the genotypic distribution revealed that out of 633 patients, 355 (56%) carried either one or two responsive alleles and thus are potential BH_4-responders. Based on the allele frequency (AF), we calculated the theoretical distribution of BH_4-responsive patients using the Hardy-Weinberg equation to be 58%, which corresponds well with the result of the genotype analysis. (Table 4).

BH_4-responsiveness in Europe, Northern China, and South Korea

The data from Germany and Northern Ireland contained genotype information, those from the remaining European countries only allele frequencies.

Based on the genotype information, 159 out of 275 (58%) potentially responsive patients were counted in Germany and 78 out of 119 (73%) in Northern Ireland. Using the Hardy-Weinberg formula a similar distribution was observed: 58% BH_4-responders in Germany and 76% in Northern Ireland (Table 4). The calculation of the expected proportion of potentially responsive patients using the Hardy-Weinberg formula gave greatly different results ranging from 17% in the Baltic countries (Estonia, Latvia, and Lithuania) and Poland to a high 79% in Spain. Relative lower numbers of potentially responsive patients were found in Eastern Europe, mainly due to the high frequency of severe PKU mutations, in particular p.R408W, whilst higher numbers of responders are expected in the genetically heterogeneous Southern Europe populations. The average of potentially responsive patients in Europe was estimated to be 55% (Table 3). Data from Northern China comprised 155 fully defined genotypes with 310 alleles whereof 106 were according to our classification assigned as responsive (Table 3). Approximately 54% of the genotypes are found to be potentially BH_4-responsive (57% using the Hardy-Weinberg formula) (Table 4). A total of 58 patients from South Korea with fully characterized mutations were used to calculate the expected frequency of BH_4-responsiveness. We found 38/116 (33%) of responsive alleles (Table 3), which leads to 31/58 (53%) potentially BH_4-responsive patients (55% according to the Hardy-Weinberg formula) (Table 4).

Genotype-phenotype correlation

Most patients tabulated in the BIOPKUdb belong to the group of MHP (42%) or mild PKU (37%). As classical PKU is frequently associated with homozygosity or compound heterozygosity for null mutations it is not surprising to find only 7% of moderate PKU and 13% of classical

PKU in the BIOPKUdb. Of 173 patients with only 1 responsive allele 39% are MHP and 38% mild PKU, while of 134 patients with 2 responsive alleles (either homozygote or compound heterozygote) 47% are MHP and 35% mild PKU. Only 14% of the patients with 1 responsive allele and 10% of the patients with 2 responsive alleles belong to classical PKU.

We compared the BIOPKUdb information with two studies performed in PKU patients and faced a similar problem. Based on the loading test performed in 74 PKU patients, Fiori et al. (Fiori et al. 2005) detected 85% of patients to be BH_4 responders. Using the genotype analysis we found five more responders (p.R261Q/p.L48S, p.L48S/p.L48S, p.L48S/p.L48S, p.L48S/p.R408W, and p.R261Q/p.R53H), raising predicted BH_4-responsiveness to 92% (+7%). Pérez-Dueñas et al. (Pérez-Dueñas et al. 2004) loaded 42 PKU patients with BH_4 and found 44% of them to be responders. We confirmed BH_4-responsiveness in 18 out of 20 patients who were initially defined as responders; two were non-responders according to the genotype (IVS2+5G>C/p.P362T and IVS4+5G>T/ IVS4+5G>T). 11 out of the 22 initially assigned non-responders were BH_4-responders according to the genotype information (p.R261Q/p.S349P, p.R243Q/p.I65T, p.L48S/p.S349P, IVS4+5G>T/p.R158Q, p.R261Q/p.R243X, p.V388M/p.S349P, IVS1+5G>T/p.R158Q, p.I65T/p.I65T, IVS4+5G>T/p.R243Q, p.I65T/IVS8+1G>A, and p.V388T/p.P279fsdelC). Thus, according to the BIOPKUdb classification by genotype, 69% (+25%) of patients described in this study are expected to be responders.

Figures and Tables

Table 1.
Summary of the BIOPKUdb mutations. Alleles frequency, location in the protein, and residual PAH activity.

Allele	Nucleotide aberration	Number of alleles (AF)	Residual activity*	Domain (CBR) (AS)
BH_4-responsive mutations				
p.A403V	c.1208C>T	56 (8.9%)	32	catalytic
p.R261Q	c.782G>A	49 (7.8%)	38.5	catalytic (CBR1)
p.Y414C	c.1241A>G	44 (7.0%)	36	tetramerization
p.A300S	c.898G>T	26 (4.1%)	31	catalytic
p.V245A	c.734T>C	26 (4.1%)	50	catalytic (CBR1) (AS)
p.L48S	c.143T>C	24 (3.8%)	39	regulatory
p.E390G	c.1169A>G	23 (3.7%)	72.5	catalytic
p.R241C	c.721C>T	20 (3.2%)	25	catalytic
p.I65T	c.194T>C	18 (2.9%)	25.3	regulatory
p.R158Q	c.473G>A	14 (2.2%)	10	catalytic
p.V388M	c.1162G>A	14 (2.2%)	27.5	catalytic
p.D415N	c.1243G>A	8 (1.3%)	93	tetramerization
p.R408Q	c.1223G>A	7 (1.1%)	49.7	catalytic
p.R243Q	c.728G>A	6 (1.0%)	23	catalytic
p.R413P	c.1238G>C	6 (1.0%)	66	tetramerization
IVS4-5c>g	c.442-5C>G	5 (0.8%)	?	intronic
p.E178G	c.533A>G	5 (0.8%)	39	catalytic
p.F39del	c.115_117delTTC	5 (0.8%)	20	regulatory
p.F39L	c.117C>G	5 (0.8%)	49	regulatory
p.R68S	c.204A>T	5 (0.8%)	87	regulatory
p.A395P	c.1183G>C	4 (0.6%)	15.5	catalytic
p.L348V	c.1042C>G	4 (0.6%)	41	catalytic
p.M276V	c.826A>G	4 (0.6%)	?	catalytic
p.P211T	c.631C>A	4 (0.6%)	72	catalytic
p.P407S	c.1219C>T	4 (0.6%)	94	catalytic
p.R241H	c.722G>A	4 (0.6%)	23	catalytic
p.Y417H	c.1249T>C	4 (0.6%)	?	tetramerization
IVS10-3c>t	c.1066-3C>T	3 (0.5%)	?	intronic
p.A104D	c.311C>A	3 (0.5%)	26	regulatory
p.A309V	c.926C>T	3 (0.5%)	44	catalytic
p.K320N	c.960G>C	3 (0.5%)	?	catalytic
p.T380M	c.1139C>T	3 (0.5%)	?	catalytic (AS)
p.V230I	c.688G>A	3 (0.5%)	63	catalytic
p.D222G	c.665A>G	2 (0.3%)	?	catalytic
p.H170D	c.508C>G	2 (0.3%)	43	catalytic
p.I94del	c.283_285delATC	2 (0.3%)	27	regulatory
p.I94S	c.281T>G	2 (0.3%)	?	regulatory
p.T92I	c.275C>T	2 (0.3%)	76	regulatory
p.V190A	c.569T>C	2 (0.3%)	110	catalytic
p.A313T	c.937G>A	1 (0.2%)	76	catalytic
p.A373T	c.1117G>A	1 (0.2%)	56	catalytic

Table 1 cont.

Allele	Nucleotide aberration	Number of alleles (AF)	Residual activity*	Domain (CBR)
p.D129G	c.386A>G	1 (0.2%)	?	regulatory
p.D338Y	c.1012G>T	1 (0.2%)	?	catalytic
p.E76G	c.227A>G	1 (0.2%)	47	regulatory
p.I269L	c.805A>C	1 (0.2%)	?	catalytic
p.L308F	c.922C>T	1 (0.2%)	49	catalytic
p.L41F	c.121C>T	1 (0.2%)	10	regulatory
p.P122Q	c.365C>A	1 (0.2%)	22	regulatory
p.P147S	c.439C>T	1 (0.2%)	?	catalytic
p.P244L	c.731C>T	1 (0.2%)	51	catalytic
p.P275S	c.823C>T	1 (0.2%)	?	catalytic
p.P314S	c.940C>T	1 (0.2%)	?	catalytic
p.S110L	c.329C>T	1 (0.2%)	?	regulatory
p.S310Y	c.929C>A	1 (0.2%)	?	catalytic
p.S87R	c.261C>A	1 (0.2%)	82	regulatory
p.R176L	c.527G>T	1 (0.2%)	31.5	catalytic
p.V177M	c.529G>A	1 (0.2%)	?	catalytic
Unclear mutations				
IVS10-11g>a	c.1066-11G>A	27 (4.3%)	?	intronic
IVS4+5g>t	c.441+5G>T	5 (0.8%)	?	intronic
IVS2+5g>c	c.168+5G>C	4 (0.6%)	?	intronic
p.F55>Lfs	c.165delT	4 (0.6%)	?	regulatory
IVS7+1g>a	c.842+1G>A	3 (0.5%)	?	intronic
p.P362T	c.1084C>A	3 (0.5%)	?	catalytic
IVS1+5g>t	c.60+5G>T	2 (0.3%)	?	intronic
IVS4+4g>a	c.441+4A>G	2 (0.3%)	?	intronic
IVS4-1g>a	c.442-1G>A	2 (0.3%)	?	intronic
IVS10+1g>a	c.1065+1G>A	2 (0.3%)	?	intronic
p.F55del	c.163_165delTTT	2 (0.3%)	?	regulatory
p.F55L	c.165T>G	2 (0.3%)	?	regulatory
p.I95_K96delinsK	c.284_286delTCA	2 (0.3%)	?	regulatory
p.R53H	c.158G>A	2 (0.3%)	?	regulatory
IVS1+5g>a	c.60+5G>A	1 (0.2%)	?	intronic
IVS10del546	?	1 (0.2%)	?	intronic
IVS2-13T>g	c.IVS2-13T>G	1 (0.2%)	?	intronic
IVS3-22g>a	c.353-22G>A	1 (0.2%)	?	intronic
IVS7+5g>a	c.842+5G>A	1 (0.2%)	?	intronic
p.A132V	c.395C>T	1 (0.2%)	?	regulatory
p.G188D	c.563G>A	1 (0.2%)	?	catalytic
p.H201Y	c.601C>T	1 (0.2%)	?	catalytic
p.I65S/p.H170Q	c.194T>G/c.510T>A	1 (0.2%)	?	regulatory/catalytic
p.I65V	c.193A>G	1 (0.2%)	?	regulatory
p.L287V	c.859C>G	1 (0.2%)	?	catalytic
p.L367>Pfs	c.1099_1100insC	1 (0.2%)	?	catalytic
p.P119S	c.355C>T	1 (0.2%)	?	regulatory
p.P275L	c.824C>T	1 (0.2%)	?	catalytic
p.P275R	c.824C>G	1 (0.2%)	?	catalytic

Table 1 cont.

Allele	Nucleotide aberration	Number of alleles (AF)	Residual activity*	Domain (CBR)
p.P281A	c.841C>G	1 (0.2%)	?	catalytic (CBR2)
p.P366H	c.1097C>A	1 (0.2%)	?	catalytic
p.R158W	c.472C>T	1 (0.2%)	?	catalytic
p.S16>XfsX1	c.47_48delCT	1 (0.2%)	?	regulatory
p.S67P	c.199T>C	1 (0.2%)	?	regulatory
p.T193I	c.578C>T	1 (0.2%)	?	catalytic
p.Y168H	c.502T>C	1 (0.2%)	?	catalytic
p.Y386C	c.1157A>G	1 (0.2%)	?	catalytic
BH_4-non-responsive mutations				
p.R408W	c.1222C>T	35 (5.6%)	1.85	catalytic
IVS12+1g>a	c.1315+1G>A	14 (2.2%)	0	intronic
p.P281L	c.842C>T	12 (1.9%)	1	catalytic (CBR2)
p.R261X	c.781C>T	7 (1.1%)	1	catalytic (CBR1)
p.S349P	c.1045T>C	6 (1.0%)	1	catalytic
p.G272X	c.814G>T	4 (0.6%)	1	catalytic
p.R252W	c.754C>T	4 (0.6%)	1	catalytic (CBR1)
p.R243X	c.727C>T	3 (0.5%)	1	catalytic
p.A259T	c.775G>A	2 (0.3%)	0.3	catalytic (CBR1)
p.E280K	c.838G>A	2 (0.3%)	1.95	catalytic (CBR2)
p.K274_Y277>Nfs	c.822_832del11	2 (0.3%)	?	catalytic
p.R176X	c.526C>T	2 (0.3%)	1	catalytic
p.Y356X	c.1068C>G	2 (0.3%)	1	catalytic
p.D222>Efs	c.663_664delAG	1 (0.2%)	1	catalytic
p.L311P	c.932T>C	1 (0.2%)	1	catalytic
p.R111X	c.331C>T	1 (0.2%)	1	regulatory
p.R270K	c.809G>A	1 (0.2%)	2.1	catalytic
p.S411X	c.1232C>A	1 (0.2%)	1	tetramerization
p.T278I	c.833C>T	1 (0.2%)	1	catalytic
p.W187X	c.561G>A	1 (0.2%)	0	catalytic

*average PAH activity (%) calculated for different cell systems; AF: allele frequency; CBR: cofactor binding region;
AS: active site
Both protein and cDNA numbers follow standard recommendations with nucleotide +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, and the initiation codon denoted codon 1. Reference sequences: GenBank cDNA = NM_000277.1; protein = NP_000268.1.

Table 2.
Distribution of BIOPKUdb mutations in different PAH domains

	Domain	Number of alleles	Percentage alleles	No. of different genotypes
all alleles	catalytic	401	63.7	66
	regulatory	90	14.3	26
	tetramerization	63	10.0	5
	intronic	75	11.9	17
	regulatory/catalytic	1*	0.2	1*
	total	**630**	**100**	**115**
responsive	catalytic	300	67.9	36
	regulatory	72	16.3	15
	tetramerization	62	14.0	4
	intronic	8	1.8	2
	regulatory/catalytic	0	0.0	0
	total	**442**	**100**	**57**
non-responsive	catalytic	86	84.3	17
	regulatory	1	1.0	1
	tetramerization	1	1.0	1
	intronic	14	13.7	1
	regulatory/catalytic	0	0.0	0
	total	**102**	**100**	**20**
unclear	catalytic	15	17.4	13
	regulatory	17	19.8	10
	tetramerization	0	0.0	0
	intronic	53	61.6	14
	regulatory/catalytic	1*	1.2	1*
	total	**86**	**100**	**38**

*patient with two mutations on the same allele

Table 3.
Allele frequencies and BH_4-responsiveness in different countries and within PAHdb.

Database*	All alleles	Responsive	Unclear	Non-re-sponsive	Not in the BIOPKUdb	Potentially responsive patients**
BIOPKUdb	630	442	86	102	-	315 (100%)
PAHdb genotype	1266	450	104	456	256	370 (58%)
PAHdb	3173	1008	323	796	1046	848 (53%)
Northern Ireland (NI)	238	121	4	88	25	90 (76%)
South Korea	116	38	17	26	35	32 (55%)
Northern China	310	106	13	49	142	88 (57%)
Europe	3655	1200	246	2023	186	1003 (55%)
Spain	177	95	43	39	0	70 (79%)
Sicily	108	55	27	26	0	41 (76%)
France	186	90	18	78	0	68 (73%)
The Netherlands	39	16	1	22	0	13 (65%)
UK (without NI)	351	139	10	181	21	111 (64%)
Belgium	195	74	12	109	0	60 (61%)
Republic of Ireland	464	172	9	258	25	140 (60%)
Germany	550	192	65	220	73	158 (58%)
Norway	201	66	2	99	34	55 (55%)
Croatia	67	21	3	43	0	18 (53%)
Denmark	281	70	17	191	3	61 (44%)
Bulgaria	46	7	15	24	0	6 (28%)
Czech Republic	213	32	9	171	1	30 (28%)
Iceland	16	2	0	10	4	2 (23%)
Romania	28	3	0	25	0	3 (20%)
Baltic States	319	29	2	288	0	28 (17%)
Poland	176	16	9	151	0	15 (17%)

*source data adapted from the PAHdb (http://www.pahdb.mcgill.ca), (Zschocke and Hoffmann 1999), and (Zschocke 2003); **calculated using Hardy-Weinberg equation.

Table 4.
Estimated and predicted BH_4-responsive genotype frequencies in different countries and within PAHdb.

Database*	Number of patients	Number of estimated responsive patients	Number of predicted responsive patients**
Germany	275	159 (58%)	158 (58%)
Northern Ireland	119	87 (73%)	90 (76%)
South Korea	58	31 (53%)	32 (55%)
Northern China	155	84 (54%)	88 (57%)
PAHdb	633	355 (56%)	370 (58%)

*source data adapted from the PAHdb (http://www.pahdb.mcgill.ca), (Zschocke and Hoffmann 1999), and (Zschocke 2003).
**calculated using Hardy-Weinberg equation

Table S1.
Most abundant genotypes in the BIOPKUdb (Supplementary Online).

Number of patients	allele 1	allele 2
8	p.Y414C	**p.R408W**
7	p.A403V	**IVS10-11G>A**
6	p.R261Q	p.L48S
6	p.R261Q	p.R261Q
6	p.A403V	p.A300S
5	p.L48S	p.L48S
5	p.R241C	p.R413P
5	p.V245A	p.R261Q
5	p.A403V	p.V245A
5	p.Y414C	**IVS10-11G>A**
4	p.R261Q	p.Y414C
4	p.E390G	**IVS12+1G>A**
4	p.A403V	p.A403V
3	p.R158Q	p.R261Q
3	p.R241C	p.R241C
3	p.V245A	**IVS10-11G>A**
3	p.A300S	**p.R261X**
3	p.A300S	**p.P281L**
3	p.A403V	**p.P281L**
3	p.A403V	**p.R408W**
3	p.A403V	**IVS12+1G>A**

Non-responsive or unknown alleles in bold

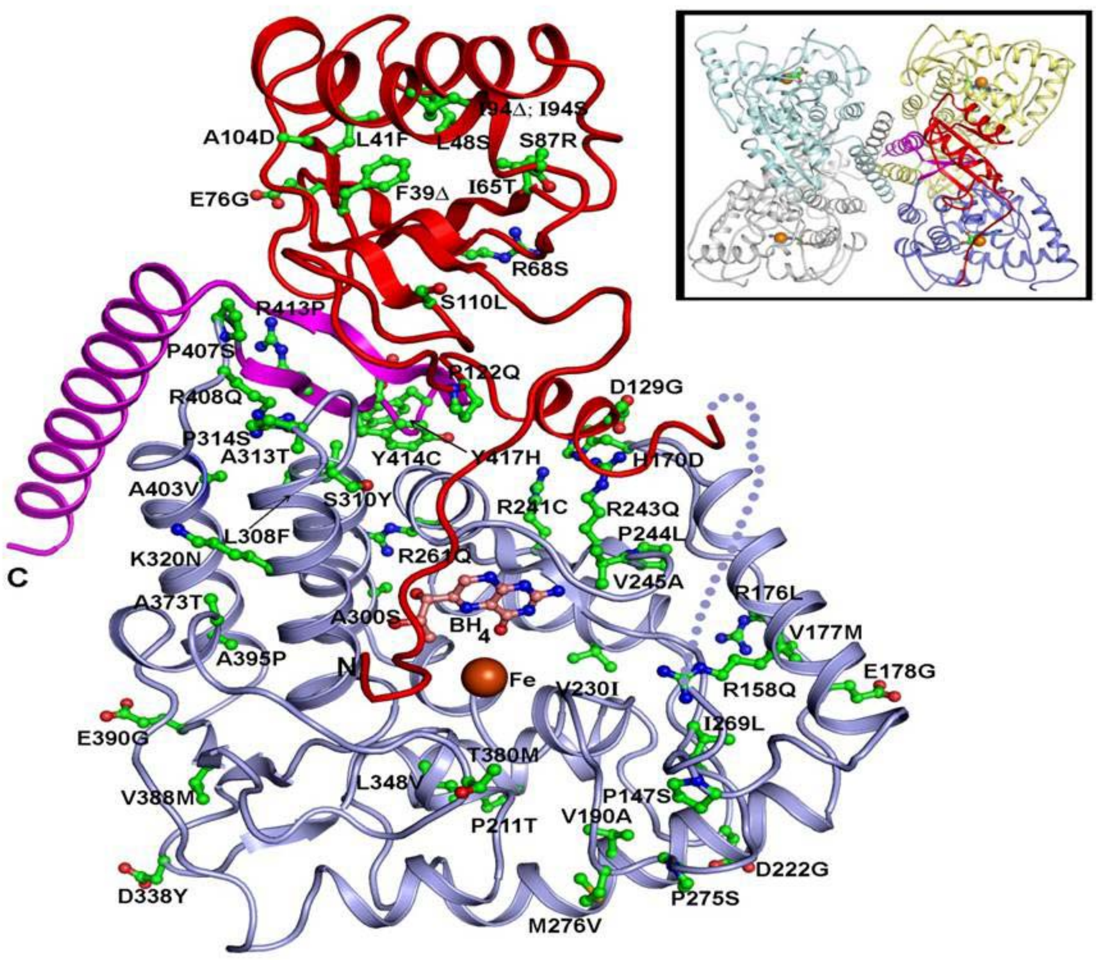

Fig. 1. BH_4-responsive mutations found in PKU patients are mapped in the 3D crystal structure of the PAH monomer. In the active site, iron atom and BH_4 cofactor are shown in red. The N-terminus starting over the active site as well as the rest of the regulatory domain are highlighted in red; catalytic domain in blue; tetramer domain is in purple. In the top right corner is the native tetramer form of the enzyme.

Discussion

We tabulated biochemical and molecular data from 315 patients with BH_4-responsive PAH deficiency in the BIOPKUdb. Alleles and genotypes defined as BH_4-responsive were compared with data previously reported for PKU patients in some European countries, in Northern China, and in South Korea. Furthermore, the PAHdb knowledgebase compiling 3171 mutant alleles from all over the world was analyzed using the BIOPKUdb information. Comparison of different databases with the BIOPKUdb allowed us to estimate both allele and genotype frequencies of BH_4-responsiveness and to compare them with data obtained from BH_4 loading tests.

Kure et al. (Kure et al. 1999) proposed that the mechanism of BH_4-responsiveness may be explained by distinct mutations in the PAH gene and that the composition of the PAH subunits may be critical for BH_4-responsiveness. Based on the BIOPKUdb information, we estimated that about 90% of BH_4-responsive PAH alleles are producing heterotetramers and depending on the type of mutation (truncated or not), the ratio between hetero and homopolymers may be the predicting factor for the responsiveness.

Erlandsen and Stevens (Erlandsen and Stevens 2001) calculated the location and proximity of some of the mutations to the BH_4-binding site in the three-dimensional structure of PAH and predicted that some of them result in mutant enzymes that are K_m variants (with a lower binding affinity for BH_4) compared to the normal phenotype. The p.V388M mutation (BIOPKUdb AF 2.2%) was shown to result in a kinetic variant form of PAH by increasing K_m values for BH_4 from 22 µM to 82 µM (Leandro et al. 2000). Further BIOPKUdb mutations reported with decreased binding affinity for BH_4 are p.F39L (AF 0.8%), p.I65T (AF 2.9%), p.R68S (AF 0.8%), p.129G (AF 02%), p.P244L (AF 0.2%), p.L308F (AF 0.2%), and p.A309V (AF 0.5%). (Aguado et al. 2007; Pérez-Dueñas et al. 2005)

The second and most probably major mechanism proposed for BH_4-responsiveness, a PAH-stabilizing effect of BH_4 (chaperon-like activity), has been confirmed by a number of investigations (Aguado et al. 2006; Erlandsen et al. 2004; Pey et al. 2004; Thöny et al. 2004). BH_4 can prevent mutant PAH from both misfolding and inactivation. This has been shown for several common BH_4-responsive mutations: e.g. p.R261Q (AF 7.8%), p.Y414C (AF 7.0%), p.I65T (AF 2.9%), p.V388M (AF 2.2%), p.R68S (AF 0.8%), p.A309V (AF 0.5%), and p.P244L (AF 0.2%) (Pey et al. 2004). In addition, BH_4 is able to enhance the wild-type enzyme activity without affecting PAH gene expression (Thöny et al. 2004). A similar effect of BH_4 on the wild-type

PAH activity was demonstrated by Kure et al. (Kure et al. 2004). They proposed that the responsiveness to BH_4 in patients with PAH deficiency is probably due to suboptimal physiological concentrations of BH_4 in hepatocytes and that enhancement of the residual activity by BH_4 supplementation may be associated with a wide range of mutations.

Initial information about the frequency of BH_4-responsiveness within patients with PAH deficiency originates from a large retrospective study of over 1900 loading tests (Bernegger and Blau 2002). Of the patients with baseline phenylalanine levels of 120-400, 400-800, 800-1200, 1200-1600, 1600-2200, and >2200 μmol/L, 65%, 74%, 33%, 17%, 0%, and 10% of patients, respectively, were defined as responders. This study, however, used data obtained with both the old 33% less active formulation of BH_4 and the new fully active 6R-BH_4 compound. In another study a total of 557 patients diagnosed with HPA were loaded with the active 6R-BH_4 (20 mg/kg) and blood phenylalanine was monitored over 8 to 24 hours (Fiege and Blau 2007). The overall prevalence of BH_4-responsiveness within patients with PKU for blood phenylalanine reduction of 20%, 30%, 40%, and 50% was 48%, 38%, 31%, and 24%, respectively, using the 8-hour modus, and 55%, 46%, 41%, and 33%, respectively, using the 24-hour modus. Using the standard 30% cut-off, BH_4-responsiveness was similar regardless of the modality in patients with mild hyperphenylalaninemia (79% to 83% responders), mild PKU (49% to 60% responders), and classical PKU (7% to 10% responders). About 46% of all HPA patients were responders 24 hours post loading (Fiege and Blau 2007). Extending the loading test to 48 hours (Fiege et al. 2005) or to one week (Shintaku et al. 2004) may detect additional patients who need longer to time to show the decreased phenylalanine levels. Several studies observed a similar high percentage of BH_4-responsiveness: 62% by Leuzzi et al. (Leuzzi et al. 2006), 46% by Mitchell et al. (Mitchell et al. 2005), 85% by Fiori et al. (Fiege et al. 2005), 38% by Bélanger-Quintana et al. (Bélanger-Quintana et al. 2005), 58% by Matalon et al. (Matalon et al. 2005), 44% by Pérez-Duenas et al. (Pérez-Dueñas et al. 2004), and 66% by Muntau et al. (Muntau et al. 2002).

Our findings reveal several features common to different patient groups; both the frequency of potentially BH_4-responsive alleles and genotypes is higher than initially assumed from the loading test studies; the BH_4-responsiveness is characterized by a substantial residual PAH activity of at least one mutant allele; most of the BH_4-responsive mutant alleles are located in the catalytic domain of the PAH subunit; and using the Hardy-Weinberg formula, BH_4-responsiveness is higher than the effective distribution of potentially BH_4-responsive genotypes.

The PAH locus knowledgebase PAHdb is the largest database with 3173 alleles reported in patients with various degrees of HPA (ca. 28% MHP, 36% mild PKU, and 56% classic PKU). Based on the alleles and genotype information, about 58% of patients are potentially BH_4-responders, an estimate higher than the information observed from the BH_4 loading test (46% responders in a population with a similar proportion of various HPA patients) (Fiege and Blau 2007). By comparing the estimated frequency of BH_4-responsiveness in different European countries, we found that BH_4-responsiveness is much higher (>75%) in southern regions of Europe with a high frequency of BH_4-responsive alleles p.R261Q, p.V388M, p.I65T, p.R158Q, or p.L48S, than in central Europe (50-70%), or in some eastern European countries (<40%) with frequent severe alleles p.R408W, p.R252W, or IVS12+1G>A (Zschocke 2003) (Table 3). Similar frequencies of BH_4-responsiveness were also estimated for the northern part of China and for South Korea (50-60% responders).

Several authors reported inconsistency of BH_4-responsiveness within the same genotypes and questioned the genotype-phenotype correlation. In addition it has been reported that some patients who responded in the single loading test did less well on a continuous supplementation with BH_4 (Lambruschini et al. 2005). Inconsistency of BH_4-responsiveness in patients with the same genotype is frequently reported, particularly in patients harboring p.L48S, p.I65T, p.R158Q, p.R261Q, and p.Y414C mutations (Desviat et al. 2004; Fiori et al. 2005; Leuzzi et al. 2006; Muntau et al. 2002; Nielsen et al. 2005; Pérez-Dueñas et al. 2004; Yildirim et al. 2007). It is also interesting that some splicing mutations are reported as BH_4-responsive (e.g. IVS4-5C>G, AF 0.8% and IVS10-3C>T, AF 0.5%). Although, as already mentioned in some of them splicing may be affected only to a minor extend and/or resulting in a partially active expressed proteins, other factors like dietary fluctuation during the test and spontaneous may be responsible for responsiveness.

In summary, from the evaluation of the data contained in the BIOPKUdb we would expect BH_4-responsiveness in about 50% of all patients with PAH deficiency. As discussed above, this number may vary from country to country and is higher than the BH_4 loading test data. It also needs to be shown to what extent genotype information can be used to predict *in vivo* BH_4-responsiveness. Different factors like initial blood Phe levels, age of the patient, BH_4 pharmacokinetics, the length of time of the BH_4 test, and possibly BH_4 stabilizers like ascorbic acid or a second mutation on the same allele may influence the outcome of the test. Thus, although there is no absolute genotype-phenotype correlation, mutation analysis provides useful information on potential non-responders in patients harboring two null alleles and may, to some extent, predict possible BH_4-responders. Further investigations are necessary in order to estimate the optimal BH_4 loading test conditions (amount of BH_4 and time of blood Phe monitoring), and genotype analysis may help to select target populations.

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Electronic-Database Information

The URLs for data presented herein are as follows:

OMIM: Online Mendelian Inheritance in Man, http://www.ncbi.nlm.nih.gov/omim (for PKU).

Chapter 5

OUTCOME AND LONG-TERM FOLLOW-UP OF 36 PATIENTS WITH TETRAHYDROBIOPTERIN DEFICIENCY

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Abstract

We describe the treatment, the clinical, and biochemical findings and the outcome of 26 patients with 6-pyruvoyl-tetrahydropterin synthase (PTPS) deficiency and 10 patients with dihydropteridine reductase (DHPR) deficiency. These are the two most common forms of the autosomal-recessively inherited tetrahydrobiopterin (BH_4) deficiency. Time of diagnosis, dosage of BH_4 and neurotransmitter precursors, folinic acid substitution, and levels of 5-hydroxyindoleacetic acid (5HIAA) and homovanillic acid (HVA) in cerebrospinal fluid (CSF) are essential parameters in the follow-up of patients. Unfortunately, treatment protocols vary greatly among patients and clinical centers, and CSF investigations and outcome assessments are not always available. Seventeen patients with PTPS deficiency and four patients with DHPR deficiency were diagnosed within 2 months after birth. In 14 patients with PTPS deficiency (54%; 9 early and 5 late diagnosed) and 2 patients with DHPR deficiency (20%; all early diagnosed) no developmental delay is observed, while in 10 patients with PTPS deficiency (38%; 6 early and 4 late diagnosed) and 8 patients with DHPR deficiency (80%; 2 early and 6 late diagnosed) development was delayed. Two PTPS-deficient patients died in the newborn period. DHPR deficiency seems to be more severe than PTPS deficiency and it is clearly the onset of treatment that determines the outcome. Our data suggest that diagnosis within the first month of life is essential for a good outcome and that low CSF5 HIAA and HVA values in CSF could be an indicator for the ongoing developmental impairment, even in the absence of neurological symptoms.

Introduction

Tetrahydrobiopterin (BH_4) deficiency represents a heterogeneous group of metabolic disorders caused by autosomal recessively inherited enzyme defects of the BH_4 synthesis or regenerating pathway (Blau et al. 2001). BH_4 is the cofactor for phenylalanine hydroxylase (PAH) (EC 1.14.16.1), tryptophan hydroxylase (TPH) (EC 1.14.16.4), tyrosine hydroxylase (TH) (EC 1.14.16.2) and nitric oxide synthase (NOS) (EC 1.14.13.39) (Kaufman 1987; Marletta 1993). It is synthesized in a three-step pathway from guanosine triphosphate (GTP) trough GTP cyclohydrolase I (EC 3.5.4.16), 6-pyruvoyl-tetrahydropterin synthase (PTPS) (EC 4.6.1.10), and sepiapterin reductase (SR) (EC 1.1.1.153). After coupling as an active cofactor to the aromatic amino acid hydroxylases, it is regenerated by pterin-4α-carbinolamine dehydratase (PCD) (EC 4.2.1.96) and dihydropteridine reductase (DHPR) (EC 1.6.99.7) (Thöny et al. 2000).

BH_4 deficiency, formerly known as malignant or atypical phenylketonuria (PKU), results in hyperphenylalaninemia (HPA), and decreased neurotransmitter and folate levels in cerebrospinal fluid. Infants affected by PTPS deficiency, the most common form of BH_4 deficiency, are frequently born small for gestational age (Smith and Dhondt 1985). In the neonatal period they may show abnormal signs such as poor sucking, impaired tone and microcephaly. Later they present characteristic extrapyramidal symptoms due to lack of dopamine in the basal ganglia (Allen et al. 1990) including truncal hypotonia, increased limb tone, postural instability, hypokinesia, choreatic or dystonic limb movements, gait difficulties, hypersalivation due to swallowing difficulties, and oculogyric crises. Ataxia, hyperreflexia, hypothermia as well as episodes of hyperthermia (in the absence of infections), drowsiness, irritability, disturbed sleep patterns, and convulsions (grand mal or myoclonic) are often seen (Ozand 1998; Blau et al. 1996). The clinical course of illness in DHPR deficiency is similar to that seen in severe forms of PTPS deficiency. In addition, extensive neuronal loss, calcifications, and abnormal vascular proliferation were noted in cortex, basal ganglia, and thalamus (Kaufman et al. 1975).

Screening tests for BH_4 deficiencies are available (urinary pterins, DHPR activity in dried blood (Arai et al. 1982), and BH_4 loading test) and performed in most developed countries in patients with HPA detected in newborn screening (Dhondt 1991; Blau 2006).

The treatment of BH_4 deficiency consists of regulating phenylalanine (Phe) levels in blood either by oral administration of BH_4 (in GTPCH and PTPS deficiency) or low Phe diet (mainly in DHPR deficiency) and substitution of the neurotransmitter precursors l-dopa and 5-hydroxytryptophan (5HTRP) (Ponzone et al. 2006). It has been shown that addition of Selegiline (MAO-B inhibitor) or Entacapone (COMT inhibitor) allowed a lower dosage of l-dopa and 5HTRP, preventing overdosing (Schuler et al. 1995, 2000; Spada et al. 2001). Folinic acid substitution is essential in DHPR-deficient patients and also in some PTPS-deficient patients with low 5-methyltetrahydrofolate (5MTHF) levels in cerebrospinal fluid (CSF).

Reports of long-term follow-up of patients with BH_4 deficiency are still scarce (Dudesek et al. 2001; Chien et al. 2001; Wang et al. 2006) and therapeutic strategies or treatment guidelines are vague and far from clinically based evidence. In this article we describe the long-term follow-up and outcome of 26 patients with PTPS deficiency and 10 patients with DHPR deficiency.

Materials and methods

Case reports

Detailed information about BH_4-deficient patients is tabulated in the BIODEF database (www.bh4.org). All patients presented with elevated blood Phe levels at newborn screening or at the time of the diagnosis. Initial biochemical data are summarized in Table 1. Mutation analysis was performed in very few patients (Table1). The clinical information is illustrated in Table 2. Additional information is included in following reports:

BIODEF ID#47 (PTPS deficiency) Despite different treatment protocols symptoms were difficult to control in this patient; motor development was delayed and expressive language development was grossly retarded. At the age of 10 years he achieved an IQ of 71 (WISC-R) (Valsasina et al. 1989).

BIODEF ID#62 (PTPS deficiency) In this boy a MAO-B inhibitor, Selegiline (0.4 mg/kg/d) was introduced at the age of 8 years and at the age of $14^{6/12}$ years a COMT inhibitor, Entacapone, (17 mg/kg/d) was added. Mental and motor development was always good, with limited episodes of tremors. He was able to attend normal school classes, to drive a car, to make sports, and at the age of 20 years he started working as a civil servant (Oppliger et al. 1997).

BIODEF ID#64 (PTPS deficiency) This boy presented with severe axial hypotonia, hypertonia, and hyperreflexia of the limbs, irritability, and myoclonic seizures in the first 10 months of life. In addition to the standard treatment, at the age of 12 years Selegiline was introduced at a dosage of 0.2 mg/kg/d and at the age of 19 years Entacapone (9 mg/kg/d) was added. He attended normal school classes with good results, without motor or cognitive disabilities. He is now 25 years old and works as a barman (Oppliger et al. 1997; Niederwieser et al.).

BIODEF ID#65 (PTPS deficiency) Despite the early treatment and possibly due to the severe fetal damage, this girl presented with hypotonia, spastic movements, irritability and convulsions in the neonatal period. Motor and intellectual development remained always severely impaired. At the age of 7 years Selegiline (0.28 mg/kg/d) was introduced, resulting in marginal clinical improvement (Oppliger et al. 1997).

BIODEF ID#89 (PTPS deficiency) This boy suffered from severe postnatal infection and showed hypoacusis in brainstem auditory evoked potentials (BAEP). His development was severely retarded despite the early treatment: at the age of 13 years he achieved an IQ of 43. In addition, he has micro adenoma of the pituitary resulting in high prolactin levels (max: 560.8 ng/ml at 21 years) which is well suppressed by Bromocriptine treatment (prolactin: 7.5–12.2 ng/ml; normal 5–15 ng/ml). At the age of 21 years (IQ 62) the young man works as an unskilled worker.

BIODEF ID#90 (PTPS deficiency) At the age of 6 years this boy started normal school and at the age of 9 years he achieved an IQ of 83. A MRI image taken at 15 years showed a 10 mm diameter lesion in the right superior temporal gyrus superior for a low grade astrocytoma which did not grow during the last two years. At present he is being neurologically examined for sporadic headache and balance disturbances. Furthermore he presents unexplained hypertension (130/90 mmHg) (Schuler et al. 1995).

BIODEF ID#96 (PTPS deficiency) This boy was born at full term from non-consanguineous parents by caesarean section because of pathologic CTG, placental insufficiency, and intrauterine dystrophy. At the age of 19 years he is mentally disabled and attends a sheltered workshop.

BIODEF ID#124 (PTPS deficiency) This boy achieved an IQ of 89 at the age of 7 years, language development was particularly delayed. At the age of 13 years he presented with an articulation disorder and showed difficulties in mathematics.

BIODEF ID#125 (PTPS deficiency) This girl was initially treated with BH_4 monotherapy (3 mg/kg/d). Neuromotor development was normal. At the age of $4^{10/12}$ years, 5HIAA in CSF dropped and 5HTRP substitution (5 mg/kg/d) was introduced, despite the absence of symptoms. Physical and mental development is totally normal.

BIODEF ID#235 (PTPS deficiency) This boy presented with cerclage of the umbilical cord, meconium aspiration, and underwent neonatal reanimation. Selegiline (0.1 mg/kg/d) was introduced at 6 years of age, resulting in an increase of neurotransmitter metabolites in CSF and folinic acid substitution was started because of low 5MTHF levels in CSF. Two years later the neurological symptoms disappeared. At the age of 14 years he achieved an IQ of 100, and he followed a normal school program.

BIODEF ID#236 (PTPS deficiency) This child developed a transient tyrosinaemia following the BH_4 loading test, which was positive (Blau et al. 1996). The neurological and mental development is normal, but she suffers from a delayed puberty due to huge hyperprolactinemia with prolactin levels decreasing only for several hours after l-dopa/Carbidopa administration. Therefore, the treatment with a slow release l-dopa/Carbidopa preparation was initiated.

BIODEF ID#251 (PTPS deficiency). At the age of 6 years this boy presented with anxiety psychosis and stuttering. After 6 months of treatment anxiety improved and stuttering was resolved. He attended normal school. At the age of 19 years he achieved an IQ of 90. During the last 5 years he developed several pigmented nevi.

BIODEF ID#281 (PTPS deficiency) At 6 years this girl achieved an IQ of 98. Motor language function was slightly impaired and the girl attended classes at a school for speech therapy for the next 4 years and then changed back to normal school. At the age of 8 years a language free test of basic intelligence (CFT-1) showed an IQ of 118. She has a persisting variable mild dystonic movement disorder particularly regarding her oral motor activity.

BIODEF ID#301 (PTPS deficiency) At the age of 1 month pterins in urine were in the normal range. For this reason this girl was treated with a phenylalanine-restricted diet. At the age of 15 months she presented with hypotonia, irritability, convulsions, developmental retardation and failure to thrive. She was diagnosed with BH_4 deficiency and treatment was initiated. Muscle tonus and development improved. At the age of 7 years she achieved an IQ of 58.

BIODEF ID#333 (PTPS deficiency) The younger brother of patient #124 was born at full term after intrauterine *Escherichia coli* infection. He died of bronchopneumonia with *Pseudomonas aeruginosa* at the age of 5 months.

BIODEF ID#343(PTPS deficiency) This girl is the HIV negative daughter of HIV positive mother and was diagnosed with mild PTPS deficiency at the age of one month. The girl was treated with BH_4 alone. She never presented any neurological symptoms and her development was always normal. She attends normal school.

BIODEF ID#346(PTPS deficiency) In this boy overall motor development was normal, but language motor function was retarded. At the age of 5 years he achieved an IQ of 79, his gait was ataxic and his speech stumbling.

BIODEF ID#350 (PTPS deficiency) This boy is the elder brother of patient ID#346. He presented with severe mental retardation at the age of 6 years, severe axial hypotonia, hypersalivation and oculogyric crisis. Six months after the start of treatment hypotonia improved and the IQ was 43. At the age of 11 years his IQ was 60.

BIODEF ID#363(PTPS deficiency) In this boy treatment was started at the age of 3 weeks. Folinic acid substitution was introduced because of low 5MTHF levels in CSF. Psychomotor development was always in the normal range.

BIODEF ID#365 (PTPS deficiency) This boy suffered from intrauterine dystrophy, perinatal asphyxia, and respiratory distress syndrome. The boy died at 7 months at home under unexplained circumstances.

BIODEF ID#372 (PTPS deficiency) This girl had a perinatal respiratory distress syndrome and hypoglycaemia. She presented with truncal hypotonia, poor sucking, and increased sweating. At the age of 5 years her developmental age was at the level of a 4-year-old child.

BIODEF ID#378 (PTPS deficiency) At the age of 8 years physical and mental development of this boy was normal and no abnormality was detected during neurological examination.

BIODEF ID#410 (PTPS deficiency) This child never presented with neurological symptoms and he developed normally.

BIODEF ID#450 (PTPS deficiency) This son of unrelated parents suffered neonatal hypoglycaemia. At the age of 2 years he had a developmental age of 18 months on the Denver developmental scale. At the age of 3 years he showed delayed language and cognitive development, and mixed (ataxic dystonic) cerebral palsy.

BIODEF ID#457 (PTPS deficiency) At the age of $2^{8/12}$ years this girl has gained a normal developmental age without any pathologic neurological findings.

BIODEF ID#478 (PTPS deficiency) This is a case of severe PTPS deficiency diagnosed at the age of 27 years in a patient with a normal IQ. The patient was diagnosed at birth with classical PKU and treated with low-phenylalanine diet until the age of 3 years, when the diet was stopped because of good metabolic control. At the age of 14 years he presented with dystonia of the mouth and neck muscles, episodes of tremor of the hands, apertured mouth, and inability to speak. These episodes were diagnosed as panic attacks. Under treatment the symptoms improved.

BIODEF ID#151 (DHPR deficiency) No neonatal screening tests were obtained in this girl. At 14 months, DHPR deficiency was diagnosed and a brain CT showed calcifications of the basal ganglia (Longhi, Valsasina, et al. 1985; Longhi, Riva, et al. 1985; Dianzani et al. 1998). The woman is now 24, has spastic tetraparesis, severe epilepsy, necessitating Phenobarbital and Valproate anticonvulsive therapy, and she is severely mentally retarded.

BIODEF ID#165 (DHPR deficiency) Neuroimaging performed in this boy at the age of 2 years revealed cerebral atrophy (Cotton et al. 1986). He is now 23 years old and shows spastic tetraparesis, epilepsy requiring anticonvulsive therapy, and mental disability.

BIODEF ID#167 (DHPR deficiency) A brain CT performed at the age of 10 months showed calcifications of the basal ganglia in this girl (Biasucci et al. 1990). Folinic acid (10 mg/d) was introduced. The symptoms and development improved. However, retardation persisted and epilepsy always required anticonvulsive medication. She developed an ataxic gait and inarticulate speech with poor vocabulary. At the age of 17 years the girl is mentally and physically disabled, needs a wheelchair, and attends a disability assistance program. She has several epileptic seizures and hypotonic crises per day.

BIODEF ID#174 (DHPR deficiency) The boy never presented with neurological symptoms and developed normally.

BIODEF ID#255 (DHPR deficiency) The girl is now 17 years old; she is severely mentally retarded and shows aggressive behavior. Epilepsy still necessitates anticonvulsive medication.

BIODEF ID#287 (DHPR deficiency) At the age of 3 years and 7 months this boy had the developmental age of 2.5 years on the Denver development scale; at the age of 7 years he achieved an IQ of 65. At the age of 11 years he attends classes in a school for mentally handicapped children.

BIODEF 334 (DHPR deficiency) Besides a slight intention tremor this boy showed no neurological symptoms. He developed normal according to his age. At 8 years ADHS was diagnosed and treated with methylphenidate.

BIODEF 339 (DHPR deficiency) In this girt treatment had been periodically discontinued by parents. At 11 years she is severely retarded and mentally disabled.

BIODEF ID#423 (DHPR deficiency) The girl was born at full term after pregnancy with gestational diabetes. The newborn period was uneventful. At the age of 4 years she had achieved an IQ of 82.

BIODEF 474 (DHPR deficiency) At the age of 2 years neuroimaging revealed diffuse calcifications of the basal ganglia in this girl. At the age of 4 years the girl continues to show serial severe convulsions despite a combined therapy with antiepileptic drugs and neurotransmitter precursors.

Biochemical methods

Diagnosis of BH_4 deficiency was established by measurement of urinary pterins (Curtius et al. 1991), DHPR activity in dried blood, and by the BH_4 loading test (Ponzone et al. 1991). Therapy monitoring was done by measuring the neurotransmitter metabolites 5-hydroxyindoleacetic acid (5HIAA) and homovanillic acid (HVA) (Blau et al. 1999), 5-methyltetrahydrofolic acid (5MTHF), and neopterin and biopterin in cerebrospinal fluid (CSF) (Blau et al. 2003), beside by clinical observation. Amino acid analysis was performed either using the classical ion exchange chromatography or tandem mass-spectrometry.

Patients' biochemical and clinical data are tabulated in the International Database of Tetrahydrobiopterin Deficiencies (BIODEF) URL: http://www.biopku.org.

Results

We retrospectively investigated the medical records of 36 patients with the diagnosis of BH_4 deficiency (26 with PTPS deficiency and 10 with DHPR deficiency). The patients originate from Germany, Italy, Hungary, Turkey, Saudi Arabia, Georgia, Afghanistan, Pakistan, Sri Lanka, and Albania and they were diagnosed between one week (#372, #450) and 27 years (#478) of age. Only 11 patients had consanguineous parents. Mean birth weight was 2841 g and 6 patients were born prematurely between 33 and 37 weeks of gestation. The results from the newborn screening for PKU, BH_4 loading test, analysis of urinary pterins, PTPS or DHPR activity, as well as initial 5HIAA and HVA concentrations in CSF are listed in Table 1. Table 2 summarizes the initial clinical information and current therapy protocols. The course of the CSF neurotransmitter metabolites 5HIAA and HVA in relation to the treatment and clinical manifestations is presented for patients with at least 9 follow-up investigations in Fig. 1 A–N.

PTPS deficiency

Among the patients with PTPS deficiency, there are 8 girls and 18 boys. Four patients from two gipsy families are siblings (#346, #350 and #124, #333). Seventeen of the PTPS-deficient patients were diagnosed within the first two months of life, 5 within the first two years (#47, #62, #64, #124, #301), 3 between 3 and 7 years (#89, #251, #350), and one at the age of 27 years (#478). Two of the PTPS patients died in the infancy period (#333 and #365). All patients were treated with BH_4 orally, doses ranging from 2 to 12 mg/kg/d. Neurotransmitter precursors were substituted in all patients except #343 who was diagnosed with the mild phenotype of PTPS deficiency. l-Dopa/Carbidopa dosage ranged from 4 to 18 mg/kg/d. Two patients who were treated with Selegiline did not receive l-Dopa (#251 and #333). 5HTRP dosage ranged from 3 to 10 mg/kg/d. Eleven patients received Selegiline at doses up to 0.4 mg/kg/d in addition to neurotransmitter precursors. Entacapone was introduced in patients #62 and #64 at 14 and 19 years of age, respectively. Folinic acid was substituted in patient #235, #363, #372, #410, and #457. Only one of the patients, who received folinic acid, is actually delayed in development. Tonus abnormalities (truncal hypotonia, hypertonia of the limbs, spasticity, and dystonic movements) were present in 20 patients, in 10 patients the abnormalities have been classified as mild and in the other 10 patients as moderate. In 4 patients (#125, #343, #378, #410) no tonus abnormalities were reported. Five patients (#64, #96, #124, #235, #350) showed sporadic oculogyric crises or myoclonic convulsions, while 5 patients (#47, #65, #89, #90, #346) presented with convulsions necessitating anticonvulsive therapy; all of the latter present at this time with moderate developmental delay. Other neurological symptoms reported in these 26 patients were irritability, tremor, ataxia, choreoathetosis, hyperthermia and hypersalivation. Three patients (#343, #378, #410) never presented with any neurological symptoms and developed completely normally. In 12 patients developmental delay was observed, 7 of them (#47, #65, #89, #96, #301, #346, #350) presenting with marked delay requiring a special environment (special school or sheltered workshop), and 3 present with slight delay allowing attendance of normal school or kindergarten (#236, #372, #450), 2 (#90, #281) were delayed in the early childhood, but do present without delay. In 12 patients no developmental delay was observed. Data from CSF analysis of 16 patients are available, 13 patients had abnormally low 5HIAA and HVA levels at diagnosis, and in 6 patients the values remained below the lower normal range. Two PTPS-deficient patients died (#333 and #365).

DHPR deficiency

Among the DHPR patients there were 6 girls and 4 boys. Four patients were diagnosed within the first month of life, while the other 6 were diagnosed at the age between 6 months and two years. Four Patients were treated with BH_4 orally, with doses ranging from 2 to 20 mg/kg/d; all patients except #167 received a phenylalanine-restricted diet. All patients received neurotransmitter substitution from the time of diagnosis. l-dopa/Carbidopa dosages ranged from 5 to 13 mg/kg/d and 5HTRP dosages ranged from 3 to 11 mg/kg/d. All patients were supplemented with folinic acid throughout the course of treatment. In four patients (#151, #165, #174, #339) Selegiline was introduced at doses up to 0.6 mg/kg/d. In Patient #174 Entacapone was given from the age of 13 years. The patients who were diagnosed early (in the first month of life) did not present with tonus abnormalities or seizures, except for a slight tremor observed in patient #334 and a slight hypotonia in the first year of life in patient #287. Other patients presented with moderate to severe hypotonia and suffer from epilepsy which is difficult to control despite anticonvulsive treatment. Two of the patients with early diagnosis developed normally according to their age (#174 and #334), one presents with slight developmental delay at present (#423), and one presents with moderate developmental delay (#287). The 6 patients whose onset of treatment was after the age of 6 months are severely retarded and not able to attend classes. CSF data are available from almost all patients (except #151). Nine patients presented with 5HIAA and HVA levels below the normal range at the time of diagnosis and in 7 of them CSF values remained low despite treatment.

Outcome

In some PTPS-deficient patients low-normal CSF values of 5HIAA and HVA correlate with a beneficial outcome (#90, #125), while in others normal CSF values of 5HIAA and HVA did not guarantee normal development (#372, #450, #96). In patients #281, #363, #236, and #378 5HIAA and HVA were in the middle range of normal and they developed normal. Patients #90 and #281 demonstrate clearly, that a developmental delay can be reversed under treatment.

Dosage of medications was very different from patient to patient, probably because adjustment of dosage was done according to the clinical presentation and personal experience. The addition of Selegiline does not seem to influence the outcome; however, in patients who receive Selegiline, dosage of l-Dopa and 5HTRP can be reduced.

In patients with DHPR deficiency it is clearly the onset of therapy which makes the difference between good and very bad outcome. There is no evidence that once severely delayed and affected child could regain normal developmental age, even under correct treatment and normal CSF values (#167). Patient #423 presented without any symptoms despite low HIAA and HVA values in CSF, but a developmental delay was noted. Similarly, patient #287 presented with occasionally low or low-normal CSF values and rarely presented with any symptoms; however, development is delayed. This indicates that in DHPR patients with early onset of treatment development is delayed even in the absence of neurological symptoms, implicating that medication should be dosed according to CSF values and not only according to presence or absence of symptoms. One exception is patient #334 whose CSF values are partly below the lower limit of normal, nevertheless, the boy developed according to his age. Generally, late diagnosis and very low CSF values led to severe mental retardation and epilepsy.

Figures and Tables

Table 1
Laboratory data of patients with BH_4 deficiency at the time of diagnosis.

BIODEF ID#	Age at diagnosis	Phe (B) newborn screening µmol/l	BH_4 Loading Test	Neo (U) mmol/mol creat.	Bio (U) mmol/mol creat.	PTPS activity in RBC µU/g Hb	5-HIAA (CSF) nmol/l	HVA (CSF) nmol/l	5-MTHF (CSF) nmol/l	Genotyp*
PTPS deficiency										
47	5 m	910	pos.	2.0	0.04	4.3	89	276	n.d.	n.d.
62	5 m	1331	pos.	27.0	0.09	1	21	158	n.d.	delV47/ delK29-S32
64	11 m	1314	pos.	13.4	<0.01	<0.1	38	134	n.d.	T67M/D136V
65	1 m	1210	pos.	27.8	<0.01	2	57	0	n.d.	T67M/?
89	3 y	1815	pos.	9.2	0.24	1.4	n.d.	n.d.	n.d.	n.d.
90	1 m 3 w	670	pos.	15.4	0.07	<0.1	163	421	22	n.d.
96	1 m	540	pos.	35.9	<0.01	0.92	32	73	n.d.	n.d.
124	6 m	420	pos.	40.0	0.56	1.3	68	344	n.d.	n.d.
125	2 w	1331	pos.	21.8	<0.01	<.01	210	592	n.d.	n.d.
235	3 w	1210	pos.	9.6	0.10	2.4	38	117	15	n.d.
236	1 m	720	pos.	40.5	<0.01	<.01	35	135	117	W115X/A12G
251	6 y	360	pos.	6.8	0.20	<.01	36	218	n.d.	n.d.
281	1 m 3 w	303	pos.	28.7	<0.01	0.96	144	293	103	E35G/T67M
301	1 y 2 m	2117	pos.	6.7	<0.01	2.5	64	253	75	n.d.
333	1 m	363	pos.	4.7	0.20	0.3	n.d.	n.d.	n.d.	n.d.
343	1 m	605	pos.	15.5	0.17	1.72	235	553	n.d.	n.d.
346	2 w	360	pos.	18.6	0.26	0.22	n.d.	n.d.	n.d.	n.d.
350	5 y 10 m	180	n.d.	4.1	0.32	0.42	n.d.	n.d.	n.d.	n.d.
363	3 w	1260	pos.	38.0	<0.01	0.21	31	81	49	n.d.
365	1 m	726	pos.	4.6	<0.01	<0.1	4.5	5	n.d.	n.d.
372	1 w	375	pos.	25.5	<0.01	0.44	138	125	12.6	n.d.
378	1 m	420	pos.	21.7	<0.01	3.1	174	361	n.d.	n.d.
410	1 m	707	pos.	5.7	<0.01	0.06	153	231	25.3	N52S/N52S
450	1 w	1031	pos.	19.0	<0.01	<0.1	307	486	68.4	T67M/T67M
457	1 m	1264	pos.	21.6	<0.01	<0.1	231	415	77.9	n.d.
478	27 y	381	n.d.	6.5	0.17	<0.1	8	25	13.7	n.d.

Table 1 cont.

BIODEF ID#	Age at diagnosis	Phe (B) newborn screening µmol/l	BH_4 Loading Test	Neo (U) mmol/mol creat.	Bio (U) mmol/mol creat.	PTPS activity in RBC µU/g Hb	5-HIAA (CSF) nmol/l	HVA (CSF) nmol/l	5-MTHF (CSF) nmol/l	Genotype*
DHPR deficiency										
151	1y 2m	1210	pos.	2.2	12.5	<0.05	n.d.	n.d.	n.d.	G17V/R221X
165	1y 1m	1210	pos.	2.8	13.2	<0.05	7	51	n.d.	H158Y/H158Y
167	7m	1797	neg.	1.9	7.9	<0.05	64	225	n.d.	n.d.
174	1m	1997	n.d.	12.6	9.2	<0.05	4	59	n.d.	n.d.
255	1y 1m	151	pos.	2.0	8.0	<0.05	15	112	n.d.	R221X/R221X
287	3w	480	pos.	5.7	3.8	<0.05	35	179	12	n.d.
334	3w	360	pos.	12.1	3.1	<0.05	21	118	37	W90X/W90X
339	6m	240	pos.	4.2	5.0	<0.05	16	130	20	n.d.
423	1m	768	pos.	9.0	3.5	<0.05	21	94	18	n.d.
474	2y 2m	847	pos.	3.0	19.5	<0.05	8	64	40.6	n.d.

Phe (B) = phenylalanine in blood; Neo (U) = neopterin in urine; Bio (U) = biopterin in urine; 5HIAA (CSF) = 5-hydroxyindoleacetic acid in cerebrospinal fluid; HVA (CSF) = homovanillic acid in cerebrospinal fluid; 5MTHF (CSF) = 5-methyltetrahydrofolic acid in cerebrospinal fluid; RBC = red blood cells; n.d.: not done.
*source BIOMDBdb (www.bh4.org) (Ponzone et al. 2004)

Table 2
Basic patients' data, incl. medication and clinical signs and symptoms.

ID	Sex m/f	Birth weight (g)	Consan-guineous parents (y/n)	Premature (y/n)	Present age (yrs)	Start of treatment	Diet (y/n)	BH_4* (mg/kg/d)	L-Dopa* (mg/kg/d)	5OHTrp* (mg/kg/d)	Selegiline (y/n)	Entaca pone (y/n)	Folinic acid	Hypotonia/ Hypertonia	Sei-zures	Other neurological symptoms	Present development al delay
PTPS deficiency																	
47	m	2830	n	n	17	5m	n	8	8	5	n	n	n	++	++	++	++
62	m	2900	n	n	20	5m	n	6	11	8	y	y	n	+	-	+	-
64	m	2920	n	n	24	11m	n	5	11	5	y	y	n	+	+	+	-
65	f	2050	n	n	20	1m	n	10	10	8	y	n	n	++	++	++	++
89	m	2080	n	y	21	3y	n	3	9	9	y	n	n	++	++	++	++
90	m	3040	n	n	14	1m 3w	n	7	6	9	y	n	n	+	++	+	-
96	m	2030	n	n	20	1m	n	5	10	8	n	n	n	++	+	++	++
124	m	2550	y	y	14	6m	n	5	5	5	y	n	n	+	+	+	-
125	f	2600	y	n	16	2w	n	5	7	7	n	n	n	-	-	+	-
235	m	3300	n	n	14	3w	n	4	4	4	y	n	y	+	+	-	-
236	f	2240	n	n	7	1m	n	11	10	10	n	n	n	+	-	-	+
251	m	3150	n	n	22	6y	n	2		10	y	n	n	+	-	-	-
281	f	2780	n	n	12	1m 3w	n	6	11	8	n	n	n	++	-	+	-
301	f	2950	n	n	8	1y 2m	n	6	10	5	n	n	n	+	-	-	++
333	m	3150	y	n	deceased	1m											
343	f	-			10	1m	n	5			n	n	n	-	-	-	-
346	m	2950	n	n	5	2w	n	4	5	5	y	n	n	++	++	++	++
350	m	-			14	6y 10m	n	5	5	5	y	n	n	++	+	+	++
363	m	2750	n	n	7	3w	n	6	10	6			y	+	-	+	-
365	m	1420	y	y	deceased	1m											
372	f	1700	n	y	6	1w	n	12	18	8	n	n	y	++	-	-	+
378	m	3000	y	y	7	1m	n	7	8	7	n	n	n	-	-	-	-
410	m	3170	n	n	6	1m	n	8	8	6	y	n	y	-	-	-	-
450	m	2120	n	n	4	1w	n	12	14	7	n	n	n	++	-	-	+
457	f	1890	y	n	2	1m	n	8	12	8	n	n	y	+	-	-	-
478	m	-	n	n	28	27y	n		5	3	n	n	n	+	-	+	-

Table 2 cont.

ID	Sex m/f	Birth weight (g)	Consan-guineous parents (y/n)	Premature (y/n)	Present age (yrs)	Start of treatment	Diet (y/n)	BH_4* (mg/kg/d)	L-Dopa*. (mg/kg/d)	5OHTrp* (mg/kg/d)	Selegiline (y/n)	Entaca-pone (y/n)	Folinic acid	Hypotonia/ Hypertonia	Sei-zures	Other neurological symptoms	Present development al delay
DHPR deficiency																	
151	f	3300	n	n	24	1y 2m	y		5	3	y	n	y	+++	+++	+++	+++
165	m	3000	y	n	23	1y 1m	y	20	9	5	y	n	y	+++	+++	+++	+++
167	f	3730	n	n	18	7m	n	2	9	6	n	n	y	++	+++	+++	+++
174	m	3900	n	n	18	1m	y	20	5	5	y	y	y	-	-	-	-
255	f		y	y	18	1y 1m	y	20	12	11	n	n	y	++	+++	++	+++
287	m	3520	y	n	12	3w	y		9	8	n	n	y	-	-	+	++
334	m	3340	y	n	10	3w	y		9	7	n	n	y	-	-	+	-
339	f	3700	n	n	7	6m	y		9	5	y	n	y	++	+++	++	+++
423	f	3570	y	n	4	1m	y		9	8	n	n	y	-	-	-	+
474	f	3300	n	n	3	2y 2m	y		13	6	n	n	y	+++	+++	+++	+++

Present age = age when data collection was stopped; Hypotonia/Hypertonia (including dystonia and spasticity): - normal tonus throughout the period of observation, + slight/seldom abnormal tonus reported, ++moderate/frequent abnormal tonus reported, +++ severe/constant tonus abnormalities; Seizures: - no signs of seizural activity the period of observation, + seldom oculogyric crises, ++ seizures requiring anticonvulsive therapy, +++ severe epilepsy; Developmental delay: - developing according to age, + slight delay, ++ moderate delay, +++ severely retarded.

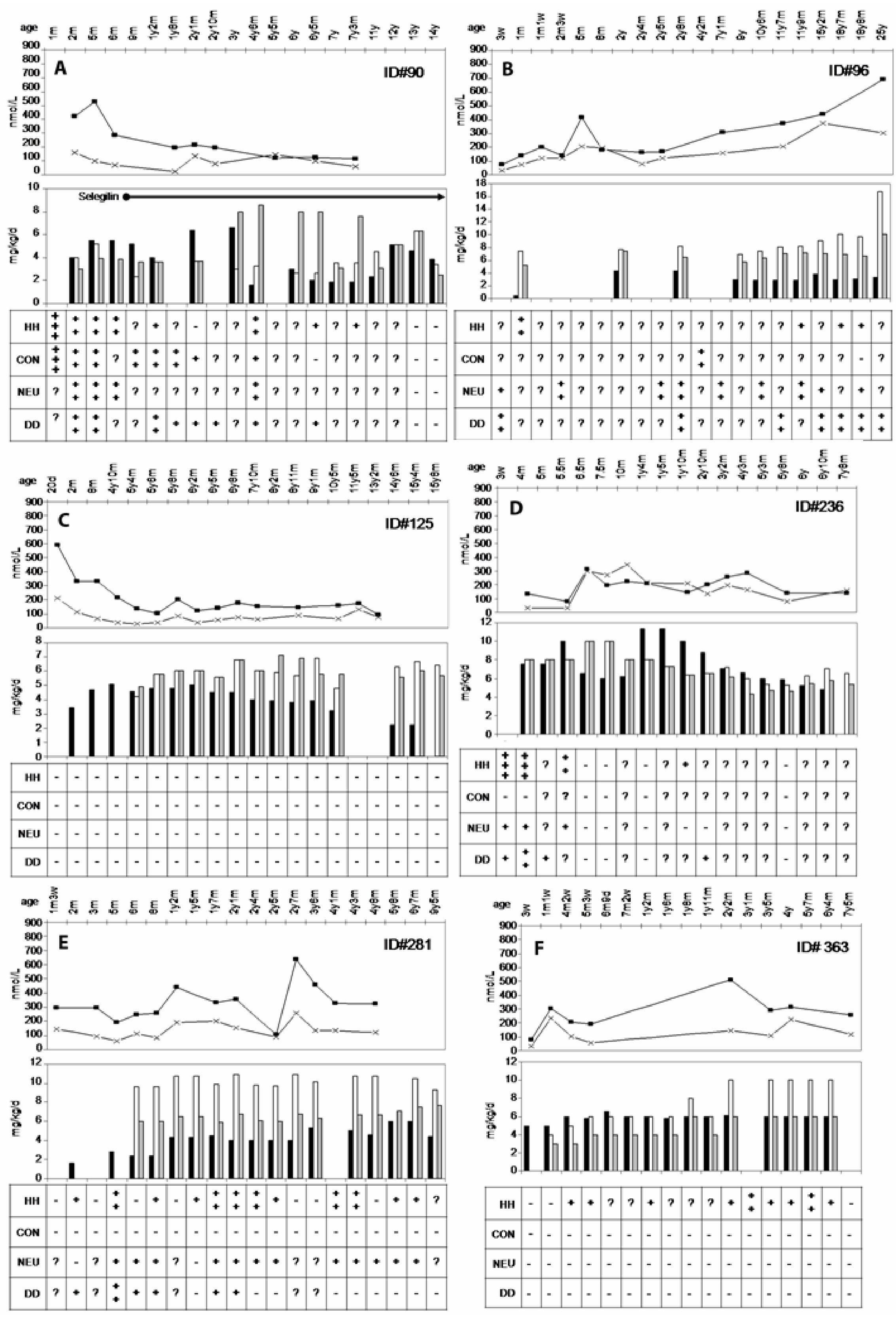

Fig. 1. A-F (continued on next page)

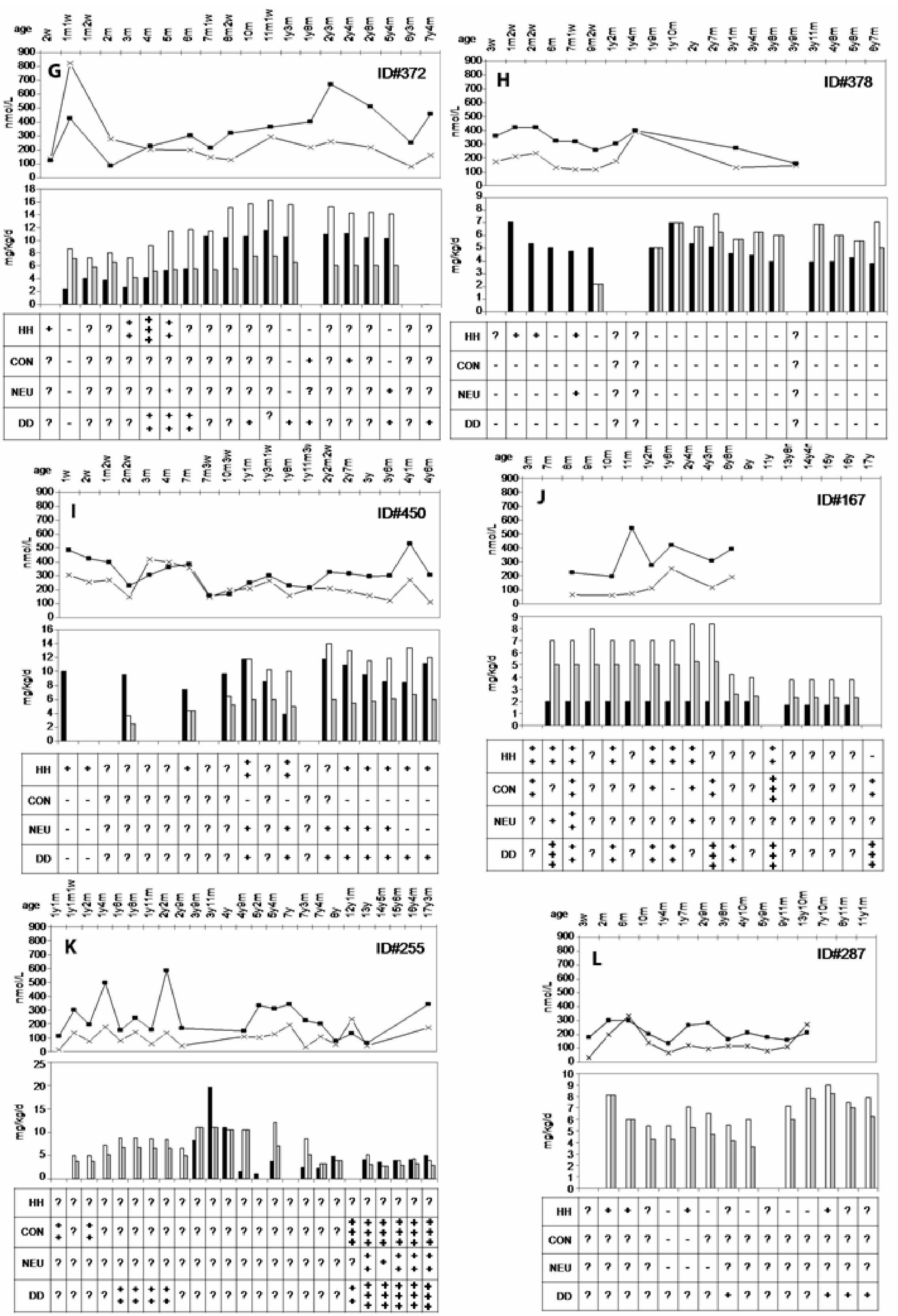

Fig. 1. G-L (continued on next page)

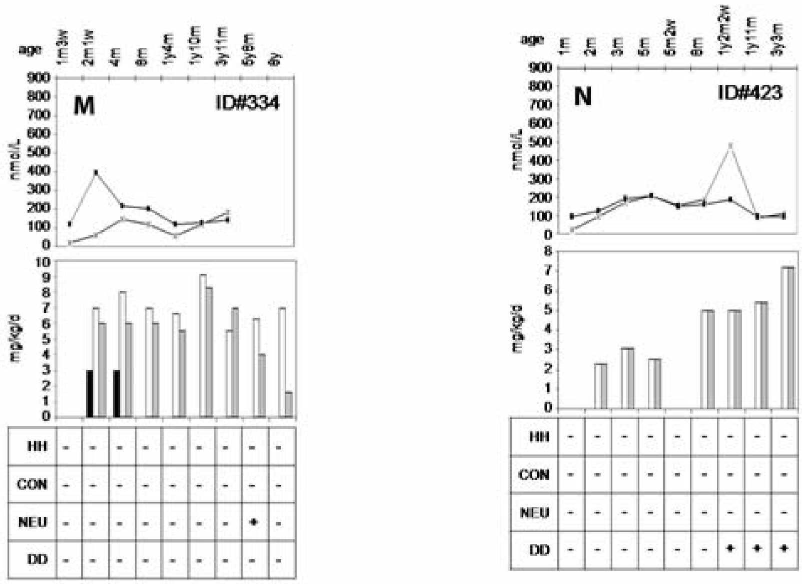

Fig. 1. Follow-up of CSF neurotransmitter metabolites 5HIAA and HVA and clinical signs and symptoms in relation to treatment modalities and age in patients with PTPS deficiency (A-I) and DHPR deficiency (J-N).
Metabolite levels: 5-hydroxyindoleacetic acid (5HIAA) -x----x----x-; homovanillic acid (HVA) -●--●--●- treatment regimen: tetrahydrobiopterin (BH_4) ■; L-Dopa/Carbidopa □; 5HTRP: 5-hydroxytryptophan □.
clinical symptoms: HH = Hypotonia/Hypertonia (including dystonia, spasticity): - normal muscle tone, + slight, ++ moderate, +++ severe; CON = convulsions: - no convulsions, + sporadic oculogyric or myoclonic crises, ++ frequent convulsions or epilepsy controlled by anticonvulsive therapy, +++ severe epilepsy, difficult to control; NEU = other neurological symptoms: - none, + one, ++ two, +++ three or more of the following: hypersalivation, tremor, ataxia, choreoathetosis, hyperthermia, irritability; DD = developmental delay: - normal child, + slight delay, attending normal classes, ++ marked delay, attending special school, working in sheltered workplace, +++ severely retarded, needing daily care; ?: not reported.

Normal values	Newborns	<1 yr	2-4 yr	5-10 yr	11-16 yr	>16yr
5HIAA (CSF) nmol/l	144-800	114-336	105-299	88-178	74-163	66-141
HVA (CSF) nmol/l	300-1000	295-932	211-871	144-801	133-551	115-488

Discussion

Today selective screening for BH_4 deficiencies is performed in most parts of the world in patients with even slight HPA detected in PKU screening. By means of a BH_4 loading test and measurement of urinary pterins and DHPR activity an exact diagnosis of the type of BH_4 deficiency can be made in the first weeks of life (Dhondt 1991; Blau 2006). This led in most countries to an early onset of therapy and prevention of the severe mental and physical disability reported of untreated patients (al Aqeel et al. 1991; Lee et al. 2006). Early diagnosis is associated with better outcome (Dhondt 2006). Treatment consisting of oral administration of commercially available synthetic BH_4 to regulate phenylalanine levels in blood and substitution of neurotransmitter precursors and folinic acid, has been shown to be effective in reducing neurological symptoms and developmental delay (Ponzone et al. 2006). However, treatment guidelines are vague; reports of long-term follow-up and outcome are rare and inconsistent. It is not yet known whether early therapy can completely prevent developmental delay in all patients with BH_4 deficiency. Since selective screening is performed, and more and more patients are detected early, it is a central concern to expand the knowledge about the course of the treated disease.

Problems of the data collection

This is a multicenter observational study, with patients spread all over Europe. The population is very heterogeneous with respect to age, some of the patients being very young. There is no general treatment or assessment scheme. It is not stated whether physical therapy, logopedia, or other supportive measures were taken. Records of IQ assessments were not always found.

PTPS

PTPS deficiency occurs in two distinctive forms: a peripheral/mild form (approximately 20%) and a central/severe form (Niederwieser et al. 1987). The mild form is commonly easy to treat with BH_4 administration alone and development is normal, as is shown in patient #343 (see case reports). The severe form necessitates neurotransmitter and sometimes folinic acid substitution in addition to BH_4 administration and the outcome differs greatly. For instance patient #65, does not present with a satisfying outcome despite early diagnosis and treatment possibly because the fetal damage, while patient #64 was diagnosed after the onset of sever neurological symptoms and today presents with a good outcome. Among the 15 early diagnosed (before 2 months of life) 9 are without developmental delay, while among 9 late diagnosed patients we found 5 without developmental delay, one of them being diagnosed in adult age (#478). In our patient population there is no clear correlation between any one factor and a good outcome. It is interesting that out of the 5 patients who received folinic acid, 4 do not present with a developmental delay. It has been reported that long-term l-Dopa/Carbidopa therapy may result in low 5MTHF levels in CSF, probably due to the methyl-group loss during the catabolism of l-dopa to vanillactic acid (Bräutigam et al. 2000). This might indicate the importance of CSF folates monitoring and folinic acid substitution in PTPS-deficient patients. This study does not allow establishing benefits of MAO-B and COMT inhibitors in the treatment of BH_4 deficiency. However, the addition of Selegiline in some cases allowed lower dosage of neurotransmitter, thus not only the on-off effects and the daily fluctuations diminished or disappeared, but probably the known side effects of the long-term treatment either. We found no clear correlation between CSF 5HIAA and HVA values and clinical outcome. This study reflects the heterogeneity of PTPS deficiency ranging from the mild form #343 over an intermediate form #125, #378, #410 to a severe form with developmental delay despite early diagnosis, administration of BH_4, and neurotransmitter substitution (#65, #90, #96, #236, #346, #372, #450). In some patients in which CSF sampling was not possible (#89, #90, #124, #235, #251, #346, #350), dopamine production was monitored by blood prolactin levels (Spada et al. 1996). Initially high blood prolactin levels normalized in all patients under standard treatment (data not shown).

Early diagnosis and correct, closely monitored therapy can reduce symptoms and prevent catastrophic outcome; however, it remains uncertain, which factor decides whether a child will experience delayed development and which not. It has been discussed that prenatal brain damage might be the cause for the most early onset neurological presentations (Dhondt 2006).

DHPR

The natural course of DHPR deficiency has been previously described (Ponzone et al. 2004). In DHPR deficiency an early diagnosis and early introduction of therapy in the first months of life is crucial, as permanent brain damage (cerebral atrophy, neuronal loss, calcification of basal ganglia) occurs early in the course of the untreated disease, and development cannot be regained. Analysis of the medical records of our 10 patients supports this, suggesting a cut-off time limit for the therapeutic intervention of one month of life. Doubtless, the diagnosis should be settled before the irreversible brain damage occurs. The course of the early-diagnosed and well-treated disease is characterized by very good control of neurological symptoms. However, it is not clear why patients #287 and #423 do present with developmental delay. Possibly the partly low HIAA and HVA values in CSF led to impaired development in the absence of other symptoms.

Acknowledgements

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Chapter **6**

GENE EXPRESSION OF BH_4 STIMULATED CELLS

Introduction

The first enzyme in the BH_4 *de novo* biosynthesis is GTPCH, mainly active in the liver. It is believed to be the rate limiting enzyme in the pathway and converts GTP to NH_2TP. In several cell types it can be stimulated by the cytokines IFN-γ and TNF-α, and on the other hand its activity can be inhibited by DAHP (Thöny et al. 2000; Xie et al. 1998). The second enzyme in the cascade is PTPS. It produces 6PTP from NH_2TP and is considered to be constitutively expressed and active in most cells. In several human cells, a splicing polymorphism had been found that leads to skipping of exon 3, indicating that alternative splicing may have a regulatory function (Thöny et al. 1994). The third and last enzyme in the pathway of BH_4 *de novo* biosynthesis is SR. This enzyme converts, under NADPH consumption, 6PTP into BH_4. Dicumarol was found being an effective inhibitor of SR. Lack of this enzyme can be partially compensated by alternative carbonyl reductases and by aldose reductase. This alternative – together with the salvage pathway – can circumvent parts of the negative effects of inborn defects in SR (namely the hyperphenylalaninemia) (Iino et al. 2003).

The homeostasis of BH_4 is controlled by a feedback regulatory circuit through interaction with a protein termed GFRP, that forms a complex with GTPCH. At high concentration, BH_4 decreases its own biosynthesis through negative feedback control, whereas on the other hand, phenylalanine has the opposite effect. Using cell cultures, it has been observed that this control mechanism not only takes place on the level of enzyme activity but also on a transcriptional level, as the expression of the genes *GCH1* and *GCHFR* are either down regulated or up regulated respectively, depending on the condition. Phenylalanine not only stimulates the biosynthesis of BH_4 by reversing the negative feedback inhibition of GTPCH, but also by increasing the mRNA level of *GCH1* (Gesierich et al. 2003).

The metabolic disorders correlated with BH_4, provide a direct insight into the metabolism of BH_4. The understanding of the metabolism is not only of academic interest but is also highly important for an early diagnosis and a possible treatment of patients. The goal of the here presented study was to further investigate the metabolism and particularly the regulation of BH_4 biosynthesis and regeneration using different cell cultures as model, based especially on the level of gene expression.

To reach this goal we examined the effects of BH_4 (either exogenously supplemented or by increasing *de novo* synthesis with cytokine induction) on enzymes and pathways which participate in the BH_4 system or which are dependent on BH_4 as a cofactor in different cell models.

We compared fibroblasts of patients, suffering from specific BH_4-deficiencies (GTPCH-deficiency and SR-deficiency) with normal fibroblasts following BH_4 supplementation. The two approaches used were stimulation of GTPCH with cytokines or adding BH_4 to culture medium. As it had been shown, that BH_4 supplemented in the culture medium is inefficiently able to increase the intracellular BH_4 pool (it is quickly excreted in form of BH_2 after being taken up by the cells), we used its precursor sepiapterin. Sepiapterin was found to be taken up quickly into cultured cells, converted to BH_2 by SR and reduced to BH_4 by DHFR (Hasegawa et al. 2005). Improved uptake and elevated BH_4 concentrations in tissues was also found *in vitro,* feeding mice with sepiapterin. Thus sepiapterin could potentially serve as an alternative to BH_4 in patients with BH_4-deficiency or BH_4-responsive PKU (Sawabe et al. 2004).

Results

Different cell lines were analysed for expression of BH_4-metabolism related enzyme activity (Table 1). Being the rate determining enzyme in the BH_4-pathway, activity of GTPCH was tested first. Certain cells, e.g. fibroblasts, need stimulation with cytokines for the induced expression of GTPCH, whereas cells of hepatic origin are supposed to express GTPCH constitutively and stimulation with cytokines usually does not lead to a significantly increased activity. Cell lines which did not express activity or only marginal levels of all the enzymes analysed in the enzyme assays, were not considered for further examination as well as cells which grew weakly. Two cell lines, expressing considerable amount of GTPCH-, PTPS-, DHPR and SR activity, were the neuroblastomal SK-N-BE and the liver carcinoma cell line

HepG2. Additionally we had decided to have a closer look at different fibroblasts of patients with a BH_4 deficiency and healthy controls.

Table 1
Enzymes activity in different cell lines

Cell Line	Enzyme	Treatment	Activity*	unit
CCF-STTG1	GTPCH	CK	0	µU/mg
	PTPS	-	0.72±0.83	µU/mg
	SR	-	201.63±156.59	µU/mg
	DHPR	-	4.23±0.85	mU/mg
Fibroblasts	GTPCH	CK	0.43±0.63	µU/mg
	PTPS	-	0.26±0.12	µU/mg
	SR	-	78.84±33.22	µU/mg
	DHPR	-	4.68±1.59	mU/mg
HepG2	GTPCH	CK	0.03±0.03	µU/mg
	GTPCH	-	0.02±0.01	µU/mg
	PTPS	-	0.38±0.5	µU/mg
	SR	-	178.69±229.45	µU/mg
	DHPR	-	6.83±0.8	mU/mg
KB	GTPCH	-	3.15±1.62	µU/mg
	PTPS	-	436.26±132.87	µU/mg
	SR	-	3.03±0.62	mU/mg
	DHPR	-	0	µU/mg
SK-N-BE	GTPCH	-	0.16±0.1	µU/mg
	PTPS	-	5.09±3.05	µU/mg
	SR	-	129.98±93.16	mU/mg
	DHPR	-	3.49±1.56	µU/mg
HHI-16	GTPCH	-	0.17±0.03	µU/mg
HHL-17	GTPCH	-	0.06±0.07	µU/mg
Huh7	GTPCH	-	0	µU/mg
THLE-3	GTPCH	-	0	µU/mg
	PTPS	-	9.19±2.12	µU/mg

Enzyme assays performed in different cell lines; Treatment: - ; untreated; CK = cytokines (TNF-α, IFN-γ); * = mean activity ± standard deviation, (n = 4-12). For more details on the cell cultures, please refer to the appropriate section in *Materials and Methods.* Page 133

Primary rat hepatocytes

Cell culture models are widely used in studies of metabolisms, toxicology, and pharmacokinetics, however due to the nature of many cell lines, being isolated and cultured from cancer tissues, enzymes of interst may not be expressed anywhere. Therefore, we decided to test primary cells. As the liver is the major site of phenylalanine catabolism, liver cells can serve as a promising model, for studies of phenylalanine respectively BH_4 metabolism. We used primary rat hepatocytes, which were cultured in a first experiment in two different kinds of culture dishes; in Falcon dishes coated with collagen or in pre-coated Sarstedt plates. Using collagen-coated dishes was reported enhancing the attachment and spreading of primary hepatocytes on the culture plate. PAH and GTPCH activity was measured on day one after isolation (prior to plating cells), on day two, three and four. PAH activity rapidly diminished and fell within 48 under 5% of the original activity. Culturing primary rat hepatocytes in presence of 100 µM sepiapterin did not change the activity substantially, neither of PAH nor of GTPCH. On day two, cells on Sarstedt plates showed a slightly higher activity (Figure 1). These plates were used in further experiments. Primary rat hepatocytes were cultured for 24 hours in presence or absence of 100 µM sepiapterin. GTPCH activity was measured before seeding the cells (-3 h), after 3 hours at the time marked by the change of WME A medium to WME B, after 18 and 24 hours (Figure 2). Measured activity declined within 18 hours significantly. Rat liver lysate was used as a control for the assay.

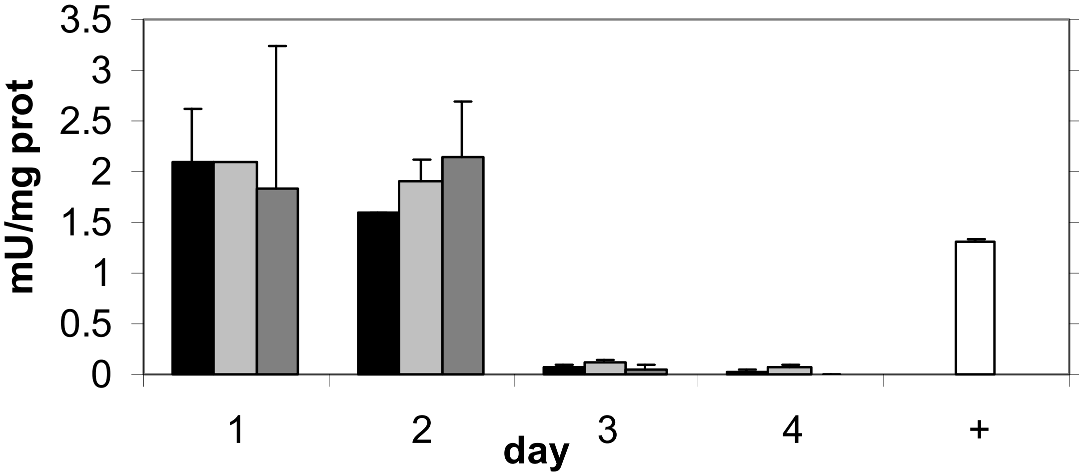

Fig. 1. PAH activity of primary rat hepatocytes on day 1 to day 4: ■ untreated cells cultured on Falcon plates; ▢ cells incubated with 100 µM sepiapterin. ▣ Untreated cells cultured on precoated Sarstedt plates; + control: rat liver; results given as mean activity and standard deviation, (n = 3).

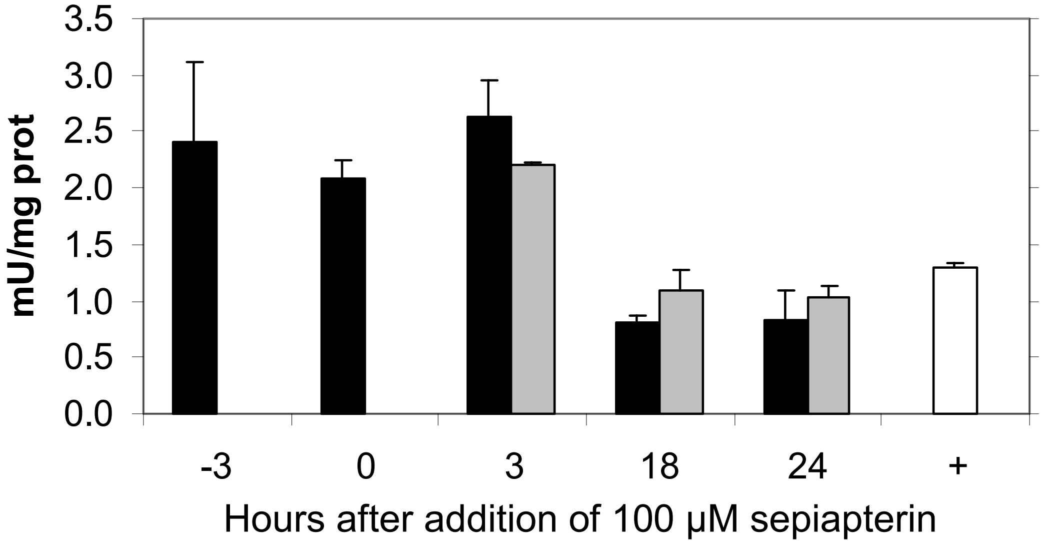

Fig. 2. PAH activity of primary rat hepatocytes cultured for 24 hours in Sarstedt plates: ■ untreated cells; □ cells incubated with 100 µM sepiapterin (supplementation with sepiapterin at 0 hours); + control : rat liver; results given as mean activity and standard deviation, (n = 3).

Primary rat hepatocytes were cultured in the presence of 100 µM sepiapterin, to see whether sepiapterin is taken up by the cells. HepG2 cells were incubated for 24 hours in 25 µM sepiapterin and its concentration in the culture medium was determined spectrophotometrically (Figure 3). Concentrations of 25 µM sepiapterin appeared to be sufficient for an incubation time of 24 hours as about 19 µM sepiapterin were consumed within this time. As sepiapterin is sensitive to light and is rapidly degraded, cells were handled at dim light and otherwise kept in the dark (Figure 4).

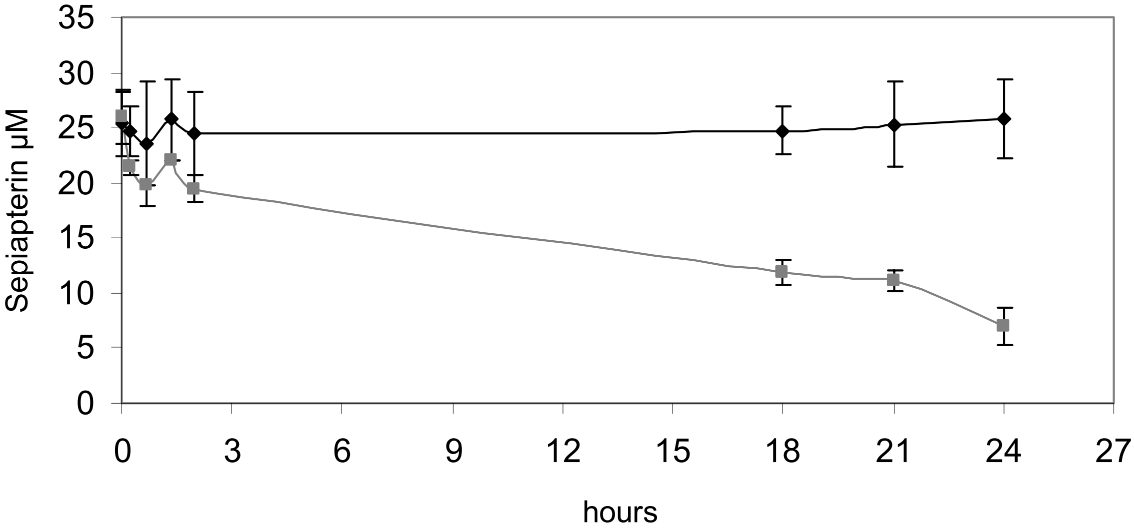

Fig. 3. Consumption of sepiapterin by primary rat hepatocytes, mean values with standard deviation: ■ 25 µM sepiapterin incubated in cell free medium; ▣ 25 µM sepiapterin incubated in the presence of primary rat hepatocytes. Δ[SP]/24 h ≈ 19 µM. Absorption at 420 nm, ε_λ = 10400 M^{-1} cm^{-1}.; results given as mean activity and standard deviation, (n = 3).

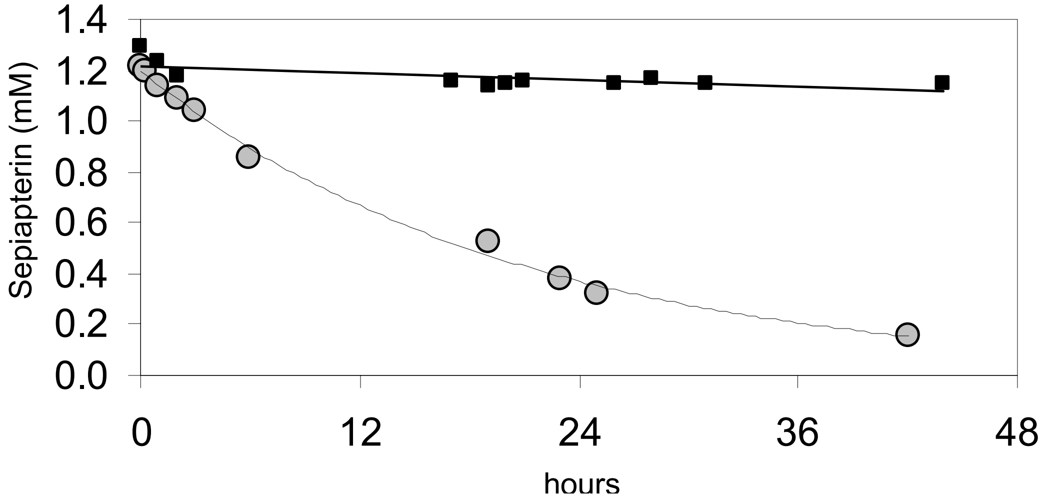

Fig. 4. Decomposition of 1.25 mM sepiapterin stock solution when left at daylight and room temperature: ■ sepiapterin solution stored in the dark at 37°C; ◙ sepiapterin solution kept at daylight and room temperature. [SP] measured in 1:20 dilution at 420 nm, ε_λ = 10400 M^{-1} cm^{-1}.

To test whether culturing primary rat hepatocytes sandwiched between two layers of collagen, forming an environment similar to the one in the liver, would increase the life span of the cells and helping to maintain the activity of the enzymes involved in BH_4 synthesis, sandwich cultures were prepared at different time points after plating the cells. In the control experiment, without collagen double layer, activity of GTPCH diminished significantly within 96 hours; sepiapterin (25 µM) had no influence on the enzyme activity (Figure 5). The same hold also true when the hepatocytes were grown in sandwich cultures. Neither, bedding the cells after 4 hours (Figure 6), nor after 24 hours after seeding (Figure 7) could stop the steadily decline of GTPCH activity. The sandwiched cells appeared to retain morphologically healthy structure for a longer period. Also, in a test for uptake of bile salts (Figure 8), indicating functional activity of the bile acid transporters, typical for functional hepatocytes, sandwiched cultures performed slightly better (Student's t test; $p \leq 0.05$). As the activity of the central enzyme of BH_4 biosynthesis could not be maintained for a longer period, which would allow investigating effects of BH_4 after repeated administration, the use of primary rat hepatocytes and sandwich cultures was not further explored.

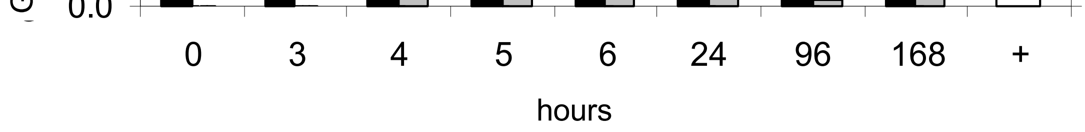

Fig. 5. GTPCH activity of primary rat hepatocytes: mean values and standard deviation, ■ untreated cells; ▢ cell incubated with 25 µM sepiapterin (supplementation at 3, 24, 48, 72, 96, 120, 144 hours); □ + control: rat liver; results given as mean activity and standard deviation, (n = 3).

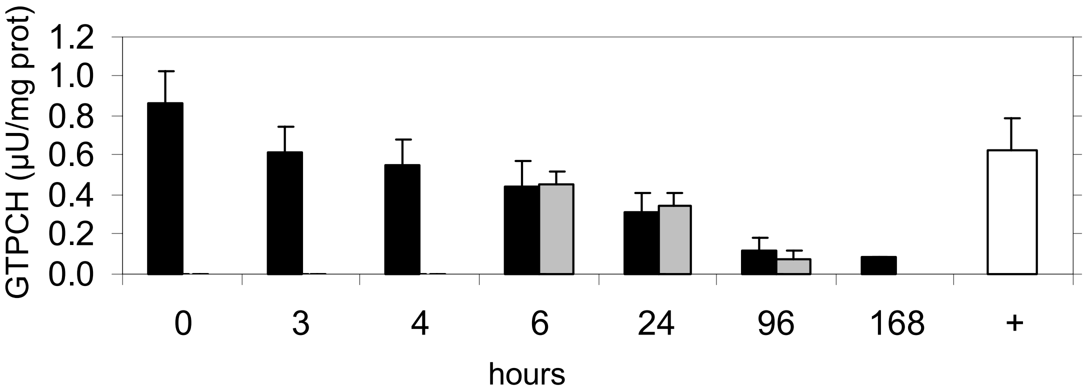

Fig. 6. GTPCH activity of primary rat hepatocytes: sandwich culture after 4 h; mean values and standard deviation; ■ control cells; ▢ cells incubated with 25 µM sepiapterin (supplementation at 4, 24, 48, 72, 96, 120, 144 hours); □ + control: rat liver. All significantly reduced after 96 h ($p \leq 0.05$); results given as mean activity and standard deviation, ($n = 3$).

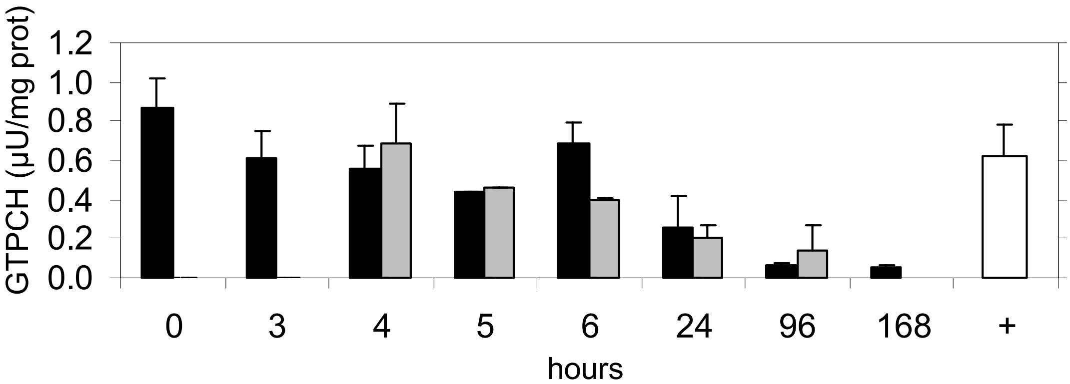

Fig. 7. GTPCH activity of primary rat hepatocytes: sandwich culture after 24 h; mean values and standard deviation; ■ control cells; ▢ cells incubated with 25 µM sepiapterin (supplementation at 3, 24, 48, 72, 96, 120, 144 hours); □ + control: rat liver. All significantly reduced after 96 h ($p \leq 0.05$); results given as mean activity and standard deviation, ($n = 3$).

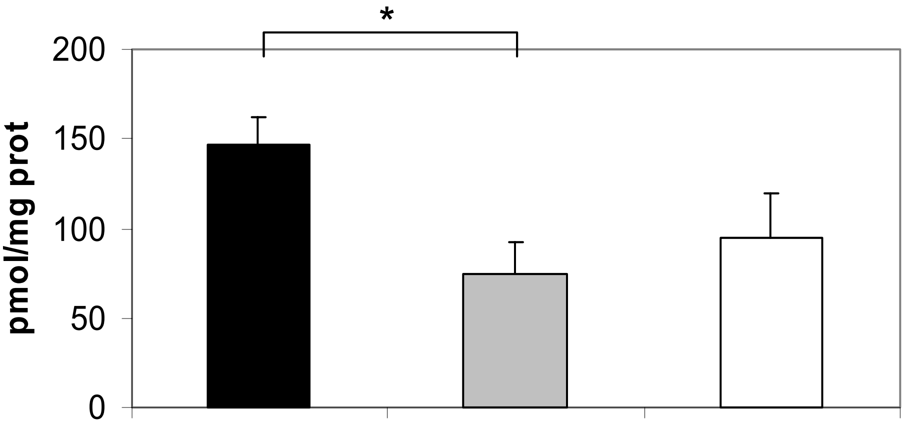

Fig. 8. Uptake study of taurocholate by primary rat hepatocytes: ■ control cells, 3 hours after seeding; ▢ controls cells, 96 hours after seeding; □ cells cultured sandwiched between two collagen layers prepared 4 hours after seeding, measured after 96 hours. At 96 hours bile acid uptake in control cells is significantly reduced compared with controls at 3 hours (*: $p \leq 0.05$). No significant reduction was observed for 96 hour-sandwich cultures; results given as mean activity and standard deviation, (n = 3).

Gene expression in fibroblasts and different cell lines

In next series of experiments, fibroblasts and other different cell lines were analysed for gene expression after either stimulation with cytokines (IFN-γ; 1250 U and TNF-α; 500 U), sepiapterin (25 µM; 100 µM), phenylalanine (2 mM), all three agents were used to increase intracellular BH_4 concentration. The first approach was used to stimulate GTPCH, the second to convert supplemented sepiapterin to BH_4, and the third to activate the GTPCH·GFRP complex via positive feedback control. Results of gene expression were acquired using Micro Fluidic Cards (mini arrays relying on RT-PCR) (Table 2).

Table 2
Micro Fluidic Card 2, gene expression assay

Gene symbol	Gene name	ABI assay number	Chromosomal location	NCBI RefSeq	Function
18S	Eukaryotic 18S rRNA	Hs99999901_s1	-		
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1	12p13	NM_002046.2	Control
ACTB	actin, beta	Hs99999903_m1	7p15-p12	NM_001101.2	Control
GCH1	GTP cyclohydrolase I	Hs00609198_m1	14q22.1-q22.2	NM_001024024.1	Synthesis
GCHFR	GTP cyclohydrolase I feedback regulator	Hs00193360_m1	15q15	NM_005258.2	Synthesis
PTS	6-pyruvoyltetrahydropterin synthase	Hs00609393_m1	11q22.3-q23.3	NM_000317.1	Synthesis
SPR	sepiapterin reductase	Hs00268403_m1	2p14-p12	NM_003124.3	Synthesis
PCBD1	pterin-4α-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha 1	Hs00165396_m1	10q22	NM_000281.2	Recycling
PCBD2	pterin-4α-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha 2	Hs00259792_m1	5q31.3	NM_032151.3	Recycling
QDPR	quinoid dihydropteridine reductase	Hs00165610_m1	4p15.31	NM_000320.1	Recycling
AKR1B1	aldo-keto reductase family 1, member B1 (aldose reductase)	Hs00739326_m1	7q35	NM_001628.2	Altern.
AKR1C3	aldo-keto reductase family 1, member C3	Hs00366267_m1	10p15-p14	NM_003739.4	Altern.
CBR1	carbonyl reductase 1	Hs00156323_m1	21q22.13	NM_001757.2	Altern.
AKR1A1	aldo-keto reductase family 1, member A1 (aldehyde reductase)	Hs00195992_m1	1p33-p32	NM_153326.1	Altern.
AKR1C1, AKR1C2	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1)	Hs00413886_m1	10p15-p14	NM_001353.5	Altern.
AKR1C4	aldo-keto reductase family 1, member C4 (chlordecone reductase)	Hs00559542_m1	10p15-p14	NM_001818.2	Altern.
DHFR	dihydrofolate reductase	Hs00758822_s1	5q11.2-q13.2	NM_000791.3	Altern.
NOS1	nitric oxide synthase 1 (neuronal)	Hs00167223_m1	12q24.2-q24.31	NM_000620.1	BH_4 req.
NOS2A	nitric oxide synthase 2A (inducible, hepatocytes)	Hs00167248_m1	17q11.2-q12	NM_153292.1	BH_4 req.
NOS3	nitric oxide synthase 3 (endothelial cell)	Hs00167166_m1	7q36	NM_000603.3	BH_4 req.
PAH	phenylalanine hydroxylase	Hs00609359_m1	12q22-q24.2	NM_000277.1	BH_4 req.
TH	tyrosine hydroxylase	Hs00165941_m1	11p15.5	NM_199292.1	BH_4 req.
TPH1	tryptophan hydroxylase 1	Hs00188220_m1	11p15.3-p14	NM_004179.1	BH_4 req.
TPH2	tryptophan hydroxylase 2	Hs00542783_m1	12q21.1	NM_173353.2	BH_4 req.

Information about the genetic transcripts detected by real-time RT-PCR. The NCBI reference sequence (RefSeq) was provided by ABI with each TaqMan Gene Expression Assay. Gene symbols and chromosomal locations are listed according to the most recent NCBI data (http://www.ncbi.nlm.nih.gov/). (*)ABI TaqMan Gene Expression Assays are under proprietary license and the exact primer and probe sequences are not available.
Function: Control = endogenous control; Synthesis = enzyme involved in BH_4 *de novo* biosynthesis; Recycling = proteins involved in the regeneration of BH_4; Altern = enzymes of the alternative and of the salvage pathway; BH_4 req. = enzyme that require BH_4 as a cofactor.

Following cell lines were analysed; Fibroblasts: FB Ko 1 and FB Ko 2 (normal control), FB SRdef (SR-deficient patient), FB GCHdef 1 and FB GCHdef 2 (GTPCH-deficient patients); liver derived cells: HepG2, HHL-17 and HHI-16 (a kind gift from A. Patel; (Clayton et al. 2005), and brain, bone marrow neuroblastoma SK-N-BE.

Gene expression analysis of single cell lines resulted in significant difference of expression only in limited cases (Figure 9). The most significant up-regulations of gene expression were gained from stimulation with the cytokines (TNF-α and IFN-γ; CK) on the *GCH1* gene and *AKR1B1*. Nevertheless a cluster of up and down-regulation was recognisable amongst other genes, after stimulation with cytokines or other agents. Expression patterns of all cell lines were compared to the endogenous control *GAPD* with reference to the chosen stimulant. The combined cytokines TNF-α and IFN-γ tended to stimulate expression of *GCH1*, *SPR* and *AKR1B1*. On the other hand the gene expression of *AKR1C1*, *AKR1C2* and *DHFR* appeared to be down regulated by cytokines. Phenylalanine generally tended to up-regulate the expression of *GCH1*, and *QDPR*; *AKR1B1* expression was repressed. The effects of 25 µM sepiapterin were not as prominent as the ones of the cytokines and changes of gene expression were smaller by an order of magnitude. Sepiapterin had rather the opposite effect on *QDPR* compared to phenylalanine and would either also lead to a repression of *CBR1* or else leave the expression level unchanged. The strongest change of expression was observed for *GCH1* especially with fibroblasts where gene expression could exceed 1000-fold up-regulation, for *AKR1B1* a maximal change of 100-fold was seen. All other genes showed in general much lower changes on the transcriptional level.

Cell line	treatment\gene	GAPDH	GCH1	GCHFR	PTS	SPR	QDPR	PCBD1	PCBD2	DHFR	AKR1A1	AKR1B1	AKR1C1;AKR1C2	AKR1C3	CBR1	PAH	NOS2A
FB Ko 1	24 h -	100	100	100	100	100	100	100	n.d.	n.d.	n.d.	100	n.d.	n.d.	100	n.d.	n.d.
FB Ko 2	24 h -	100	100	n.d.	n.d.	100	100	100	n.d.	n.d.	n.d.	100	n.d.	n.d.	100	n.d.	n.d.
FB SRdef	24 h -	n.d.	100	n.d.	100	100	100	100	n.d.	n.d.	n.d.	100	n.d.	n.d.	100	n.d.	n.d.
FB GCHdef 1	24 h -	100	100	100	100	100	100	100	n.d.	n.d.	100	100	100	n.d.	100	n.d.	n.d.
FB GCHdef 2	24 h -	100	100	100	100	n.d.	100	100	100	100	n.d.	100	100	100	100	n.d.	n.d.
HHL 17	24 h -	100	100	100	100	100	100	100	100	100	100	100	100	100	100	n.d.	n.d.
HHI 16	24 h -	n.d.	100	100	100	100	100	100	100	100	100	100	n.d.	100	100	n.d.	n.d.
HepG2	24 h -	100	100	100	100	100	100	100	n.d.	n.d.	n.d.	100	n.d.	100	100	100	100
SK-N-BE	24 h -	100	100	n.d.	100	100	100	100	n.d.	n.d.	n.d.	100	n.d.	100	100	n.d.	n.d.
FB Ko 1	24 h CK	89	11524	50	106	77	47*	111	n.d.	n.d.	n.d.	303*	n.d.	n.d.	87	n.d.	n.d.
FB Ko 2	24 h CK	122	10195*	n.d.	n.d.	193	33	138	n.d.	n.d.	n.d.	613	n.d.	n.d.	79	n.d.	n.d.
FB SRdef	24 h CK	n.d.	8937*	n.d.	44	138	30	203	n.d.	n.d.	n.d.	975*	n.d.	n.d.	29	n.d.	n.d.
FB GCHdef 1	24 h CK	107	9514	62	60	170	44	65	n.d.	n.d.	80	746*	47	n.d.	96	n.d.	n.d.
FB GCHdef 2	24 h CK	83	628*	234	42	n.d.	68	72	177*	77	n.d.	83	73	31	79	n.d.	n.d.
HHL 17	24 h CK	130	530*	91	25	162	109	122	59	62	113	145	30	108	176	n.d.	n.d.
HHI 16	24 h CK	n.d.	1179*	12	163	67	106	84	51	53	142	244	n.d.	129	n.d.	n.d.	n.d.
HepG2	24 h CK	107	149*	96	79	115	102	105	n.d.	n.d.	n.d.	151*	n.d.	65	123	95	60
SK-N-BE	24 h CK	131	137	n.d.	165	144	139	95	n.d.	n.d.	n.d.	113	n.d.	n.d.	258	n.d.	n.d.
HHL 17	24 h Phe	99	74	n.d.	25	151	98	134	69	69	76	78	36	100	75	n.d.	n.d.
HHI 16	24 h Phe	n.d.	101	18	169	65	118	97	207	102	72	108	n.d.	109	42	n.d.	n.d.
FB Ko 1	6 h Phe	82	161	72	89	73	126	138	n.d.	n.d.	n.d.	58*	n.d.	n.d.	50	n.d.	n.d.
FB Ko 2	6 h Phe	97	190	n.d.	n.d.	95	141	48	n.d.	n.d.	n.d.	88	n.d.	n.d.	161	n.d.	n.d.
HepG2	6 h Phe	102	134	106	90	109	92	112	99	100	87	107	104	102	149	86	166
FB Ko 1	24 h SP	92	113	60	131	74	106	78	n.d.	n.d.	n.d.	82	n.d.	n.d.	74	n.d.	n.d.
FB Ko 2	24 h SP	90	131	n.d.	n.d.	126	85	56	n.d.	n.d.	n.d.	89	n.d.	n.d.	95	n.d.	n.d.
FB SRdef	24 h SP	n.d.	235	n.d.	34	50	34	123	n.d.	n.d.	n.d.	74	n.d.	n.d.	28	n.d.	n.d.
FB GCHdef 1	24 h SP	65	53	46	74	93	101	66	n.d.	n.d.	43	133	31	n.d.	71	n.d.	n.d.
FB GCHdef 2	24 h SP	96	164	142	27	n.d.	59	126	175*	79	n.d.	108	39	242	56*	n.d.	n.d.
HHL 17	24 h SP	103	67	78*	43	149	102	154	107	112	125	103	118	132	96	n.d.	n.d.
HHI 16	24 h SP	n.d.	34	6	146	64	82	62	79	80	63	181	n.d.	88	66	n.d.	n.d.
HepG2	24 h SP	101	89	100	50	102	73*	115*	n.d.	n.d.	n.d.	88	n.d.	96	104	108	166
SK-N-BE	24 h SP	77	67	n.d.	97	716	67*	123	n.d.	n.d.	n.d.	221	n.d.	225	55	n.d.	n.d.

Fig. 9. Gene expression study of different cell cultures. Relative expression numbers (%); numbers marked with (*): statistically significantly modified gene expression (Student's t-test; $p \leq 0.05$); green, turquoise colours: up-regulated expression; red and yellow colours: down-regulated gene expression, grey: nearly unchanged expression levels, n.d.: not detected. For percentage and colour code, refer to the list on the right hand. Treatment: 24 h - = untreated control cells (were set to 100% expression and all other expression numbers were relative to them); 24 h CK = cells supplemented for 24 hours with cytokines (TNF-α and IFN-γ) 24 h Phe resp. 6 h Phe = cells incubated with 2 mM phenylalanine for 24 hours, respectively 6 hours prior to harvesting. 24 h SP = cells incubated with sepiapterin (25 μM). For more details on the cell cultures, please refer to the appropriate section in *Materials and Methods*.

%
< 50
50-90
90-110
110-200
> 200
n.d.

In parallel to the gene expression profile, the main metabolites, neopterin and biopterin, were measured in the cells and in the cell culture medium, and enzyme assays for GTPCH, the rate limiting enzyme of the biosynthesis of BH_4, known to alter activity upon cytokine stimulation, and SR, the principle enzyme in reduction of exogenous sepiapterin to BH_2, were performed. In contrast to the PTPS and GTPCH which were measured close to the detection limit, SR assay resulted in better reproducible measurements, with smaller standard deviations than the two other assays. As expected, stimulation with cytokines, lead to the strongest change in the metabolism of BH_4, especially in fibroblast which normally do not show GTPCH activity but upon stimulation with cytokines, enzyme activity became measurable. Further, intracellular concentration of biopterin and neopterin were increased upon stimulation. In unstimulated cells these two metabolites generally remained undetected (Table 3). Supplementation of the cell culture medium with sepiapterin, in concentrations between 25 μM and 100 μM had no influence on the activity of neither GTPCH nor SR. However, addition of sepiapterin to cultured cells led to a massive increase in intracellular biopterin (Table 3). The reason for this is fast uptake of sepiapterin and its conversion to BH_2, and subsequent reduction to BH_4. Incubations with phenylalanine did not lead to a significant change of the metabolism.

Table 3
GTPCH and SR activity assay and intracellular and extracellular neopterin and biopterin concentrations in fibroblasts and HHL-17, HHI-16, and HepG2 cells cultured under different conditions.

Cell line	Treatment	x̄±SD GTPCH µU/mg prot	x̄±SD neopterin pmol/mg	x̄ ±SD biopterin pmol/mg	x̄ ±SD ex neo pmol/mL	x̄ ±SD ex bio pmol/mL	x̄ ±SD SR µU/mg prot
FB Ko 1	24h -	0	0	0	10.1±9.7	17.5±5.8	150.3±41.8
	24h SP	0	0	183.1±88.6*	10.5±11.9	52.6±10	167.6±65.9
	24h CK	0.46±0.21*	7.2±7.8*	111.2±37.4*	11.3±2.9	33.4±7.1*	157.6±40
	6h 2mM Phe	0	3.1±4.3	0	n.d.	n.d.	n.d.
	24h SP100	0	2±2.8	411.6±25	n.d.	n.d.	n.d.
FB Ko 2	24h -	0	0	0.6±1.1	5.8	21.2	152.4±0.9
	24h SP	0	0	129.3±74.5*	7.2	73.1	215.1±8.5
	24h CK	0.61±0.45	22.1±22.1*	102.1±24.2*	11.2	29.4	189.4±11.4
	6h 2mM Phe	0	0	0	n.d.	n.d.	n.d.
	24h SP100	0	0	340.5±21.8*	n.d.	n.d.	n.d.
FB SRdef	24h -	0	0	2.2±4.9	8.9	21.4	0
	24h SP	0	0	8.8±10.5	3.5	31.4	0
	24h CK	3.35±0.39	101.2±74.6*	16.6±20.6	n.d.	23.6	0
FB GCHdef1	24h -	0	0	0	16.8±11.8	16±3	100.8±30.6
	24h SP	0	0.4±0.9	208.1±29.9*	29.3±21.7	80.9±2.2*	109.2±2.9
	24h CK	0.03	2.4±4.1	44.9±42.1*	20.5±14.7	37.8±1.7*	103±14.4
FB GCHdef2	24h -	0.05	1.2±2.6	3.4±6.3	16.7±5.3	16.4±2.7	88±2.4
	24h SP	0.04	1.4±2.8	578.5±321.7*	34±0	216±0	58.5±8.6
	24h CK	0.15	16.9±9.9*	40.4±16.4*	17.3±3.8	29.6±6.4	62.7±6.4
FB GCHdef3	24h -	n.d.	0	0	11.5±0	23±0	n.d.
	24h SP	n.d.	1.2±1.7	2.6±3.7	6.4±0	37.4±0	n.d.
	24h CK	n.d.	0	6.5±0.2*	10±0	37±0	n.d.
HHL-17	24h -	0.12±0.17	3.7±5	7.9±10.9	16.9±20.9	27.6±27.5	210.6±36.3
	24h SP	0.17±0.19	0	435.4±225.3*	121.9±111.9	1181.4±1200.6	234±27.5
	24h CK	0.26±0.24	5.3±1.6	19.7±2.4*	12.8±15.1	32.4±19.7	173.4±35.2
	6h 2mM Phe	n.d.	1.7±2.4	2±2.8	n.d.	n.d.	n.d.
	24h SP100	n.d.	0	1746.3±51.4*	n.d.	n.d.	n.d.
HHI-16	24h –	n.d.	0	4.3±6.1	n.d.	n.d.	n.d.
	24h SP	n.d.	0	0	n.d.	n.d.	n.d.
	24h CK	n.d.	4.3±0.2*	2046.3±86.8*	n.d.	n.d.	n.d.
	24h Phe 2mM	n.d.	0	44.9±5.9	n.d.	n.d.	n.d.
HepG2	24h -	0.29±0.11	5.8±6.3	9±1.6	33.7±7.2	100.9±73.4	399.5±23.6
	24h SP100	0.22±0.07	546.8±39.2*	3017.5±2276.5*	891.2±421.7	5735.3±595.1*	404±5.3
	24h CK	0.33±0.10*	4.3±0.1	13.3±2.5	31.1±4.5	98.2±30.9	425.8±5.7
	6h 2mM Phe	0.29±0.01	3±2.6	11.9±1.3	27±0.8	84.1±76.1	n.d.
SK-N-BE	24h -	0.35±0.16	0	82.4±4.7	8±2.4	141.8±88.8	110.9±12.5
	24h SP	0.32±0.2	0.5±0.9	844±135.8*	89.6±126.8	1130.9±1493.8	125.8±17.7
	24h CK	0.32±0.08	1.2±1.7	69±6.6*	19.8±28	424±528.7	118.8±28.8

Treatment: 24h - = untreated control cells; 24h CK = cells supplemented for 24 hours with cytokines (TNF-α and IFN-γ); 24h SP = cells incubated for 24 hours in 25 µM sepiapterin, respectively 24h SP100, incubated with 100 µM sepiapterin; 24h Phe = cells incubated in 2 mM phenylalanine for 24 hours. ex bio resp. ex neo = extracellular biopterin/neopterin measured in used culture medium samples. Results given as mean values ± standard deviation. For details on cells, see *Materials and Methods*.

Gene expression analysis in patients' fibroblasts

In a next step we investigated fibroblasts, to explore whether there were differences in the regulation of genes in fibroblasts of BH_4 deficient patients compared with healthy controls. We selected seven different fibroblast samples of patients with a known GTPCH-deficiency, one with SR-deficiency; and three samples from healthy subjects. Using conventional RT-PCR we focused on the expression of *GCH1* and *GCHFR* after stimulation with cytokines (IFN-γ: 1250 U and TNF-α: 500 U). In all samples that we analysed a strong induction of GTPCH expression following cytokine treatment was observed (Figure 10). Expression levels and enzyme activity did not necessarily correlate, as also GTPCH deficient cells showed a high induction of mRNA production which did not lead to measurable amounts of functional enzyme. Cytokines seemed to reduce *GCHFR* production in the fibroblasts, at least in the BH_4 deficient cells, where levels of *GCHFR* were significantly reduced compared to untreated cells (Figure 10). In the normal cells, no significant reduction or increase of the gene expression was found. Reduction of *GCHFR* levels along with induction of *GCH1* appears to be another mechanism by which GTPCH activity seems to be regulated in the cells. To show whether the same holds true for non-BH_4-defiecient fibroblasts further investigations need to be done.

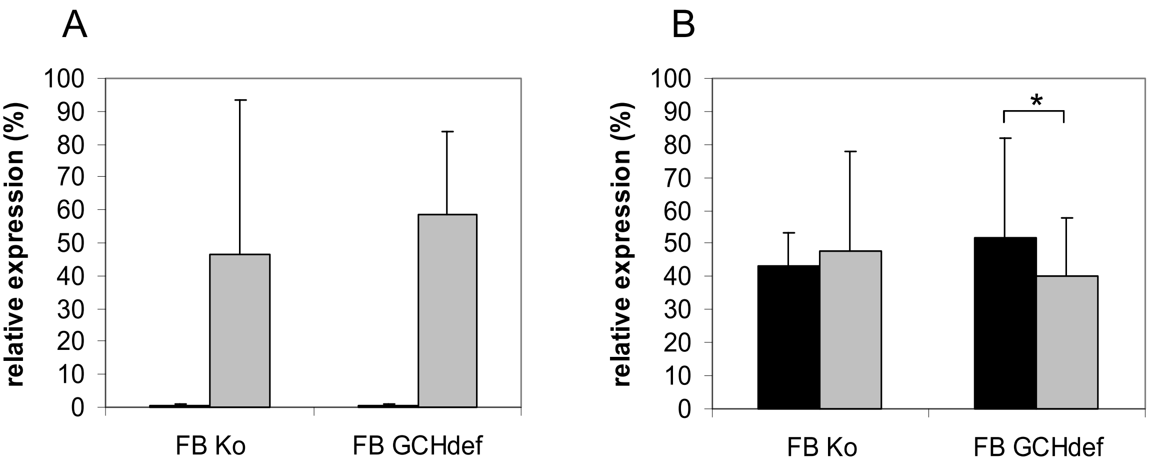

Fig. 10. Relative gene expression of **A)** *GCH1* in different human fibroblast of patients with BH_4 deficiency; (FB Ko = normal control (n = 6), FB GCHdef: = GTPCH deficiency (n = 16)), results given as mean activity and standard deviation: ■ unstimulated cells (no cytokines); □ incubated for 24 hours in presence of cytokines (IFN-γ: 1250 U and TNF-α: 500 U). Cytokines stimulated GTPCH expression significantly ($p \leq 0.01$). **B)** Relative expression of the gene *GCHFR* in different human fibroblast of patients with BH_4 deficiency; (FB Ko = normal control (n = 6), FB GCHdef: = GTPCH deficiency (n = 16)): ■ unstimulated cells (no cytokines); □ incubated for 24 hours in presence of cytokines (IFN-γ: 1250 U and TNF-α: 500 U). Expression of *GCHFR* in BH_4 deficient patient is significantly reduced after stimulation with cytokines ($p \leq 0.05$). For details on cell types; refer to section *Materials and Methods*.

Gene expression profile in HepG2 cells

PAH is almost exclusively a hepatic enzyme and thus, liver cells are most promising for the study of phenylalanine catabolism and also for BH_4 metabolism. We selected the HepG2 cell line, which provided active DHPR, SR, PTPS, PAH and GTPCH enzyme. The latter was also constitutively expressed. The cell culture medium was supplemented either with cytokines (IFN-γ; 1250 U and TNF-α; 500 U), sepiapterin (100 µM), phenylalanine (2 mM), all three expected to increase intracellular BH_4 levels; or dicumarol (100 µM) or DAHP (10 mM), both with inhibiting effect on the BH_4 biosynthesis, and combinations of these. Gene expression was studied after a 24-hour incubation. Phenylalanine was added 6 hours prior to cell harvesting. Dicumarol is a know inhibitor of SR activity and DAHP inhibits BH_4 synthesis at the level of GTPCH.

The different agents had variant effects on the genes. The gene expression of *AKR1B1* (the gene encoding for aldose reductase, AR) was significantly induced after cytokine treatment but also after supplementation with the GTPCH-inhibitor DAHP and by dicumarol. These results are rather contradictory. The gene expression of the aldo-keto reductase *AKR1C3* was induced by dicumarol and down regulated by any combinations containing cytokines, except the mixture of dicumarol and CK. For the carbonyl reductase gene *CBR1* even more controversial results were observed. Its transcription was either induced by CK, phenylalanine or DAHP but any combinations of those had rather a tendency for the opposite effect, even though no significant change was found with combined treatment. Dicumarol alone or in combination with other agents significantly induced expression of *GCH1*. *GCHFR* gene expression was significantly down regulated by virtually all combinations of the reagents used, except when treated with the mixture DAHP/SP. *PCBD1* expression was slightly induced by sepiapterin and somewhat stronger by a cytokine/phenylalanine mixture. Concerning *PTS*, the gene expression was significantly down-regulated by sepiapterin and most mixtures containing it. *QDPR* expression was reduced by several agents, namely by sepiapterin, DAHP and combinations of these two. The expression of the gene encoding sepiapterin reductase, *SPR*, was induced by DAHP and mixtures of it. Surprisingly it was induced by the notedly inhibitor of SR, dicumarol. Dicumarol had also a distinct effect on several other genes. Especially the expression of iNOS and eNOS were markedly induced by dicumarol alone and mixtures of the other reagents. The significance of this finding is not clear. Increased expression of eNOS and iNOS is often mentioned in the context of liver or hepatocyte damage (Koti et al. 2005; McNaughton et al. 2002). In adipocytes, NOS activity was found to be reduced by dicumarol, most likely due to inhibition of the cofactor biosynthesis (BH_4). In this study, cytotoxicity, as reason for the observed effects of dicumarol, was ruled out (Linscheid et al. 2003). Results of the experiments are summarised in Figures 11-16.

	AKR1A1	AKR1B1	AKR1C1/2	AKR1C3	AKR1C4	CBR1	DHFR	GCH1	GCHFR	NOS2A	NOS3	PAH	PCBD1	PCBD2	PTS	QDPR	SPR	TPH1
A	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
B	107	155	90	79	84	123	99	128	108	98	101	100	107	91	72	104	111	108
C	102	90	76	97	82	99	95	84	106	161	77	106	115	98	57	74	100	61
D	87	107	104	102	175	149	100	134	106	166	202	86	112	99	90	92	109	116
E	80	168	104	100	209	149	68	124	114	271	65	116	100	109	64	75	165	131
F	92	176	149	158	200	129	32	218	99	254	1139	123	90	99	93	116	145	147
G	78	137	90	98	167	128	51	81	102	209	88	110	88	123	71	61	151	106
H	50	277	84	56	82	96	39	117	69	148	28	67	99	85	75	53	123	61
I	74	144	67	81	78	121	69	119	68	319	72	99	106	115	98	59	162	58
J	67	108	95	106	55	100	41	152	67	189	554	117	87	75	138	67	83	90
K	73	232	116	112	84	81	44	151	64	183	443	93	89	52	114	64	96	45
L	63	172	119	106	102	96	54	142	87	311	529	110	87	81	85	74	107	49
M	97	154	56	66	76	88	127	106	59	103	74	80	143	77	51	66	103	53
N	85	138	88	104	133	86	107	97	77	336	80	103	109	105	42	73	92	26
O	85	158	47	68	68	80	137	93	69	132	79	79	90	88	44	71	83	96

Fig. 11. Gen expression of HepG2 cells: A = untreated contr., B = 24 hour incubation with cytokines; C = 24h incubation with 100 µM sepiapterin; D = 24 hour incubation in DMEM with addition of 2 mM phenylalanine 6 hours before harvesting; E = 24 hour incubation with 10 mM DAHP; F = 24 hour incubation with dicumarol; G = 24 hour incubation of a mixture of C and E (SP/DAHP); H = mixture of B and E (CK/DAHP); I = mixture of D and E (Phe/DAHP); J = mixture of C and F (SP/Dic); K = mixture of B and F (CK/Dic); L = mixture of D and F (Phe/Dic); M = mixture of B and D (CK/Phe); N = mixture of C and D (SP/Phe); O = mixture of B and C (CK/SP); ☐ significantly reduced expression compared to A, ($p \leq 0.05$); ☐ significantly increased expression compared to A, ($p \leq 0.05$); ☐ reduced expression, respectively ☐ increased expression.

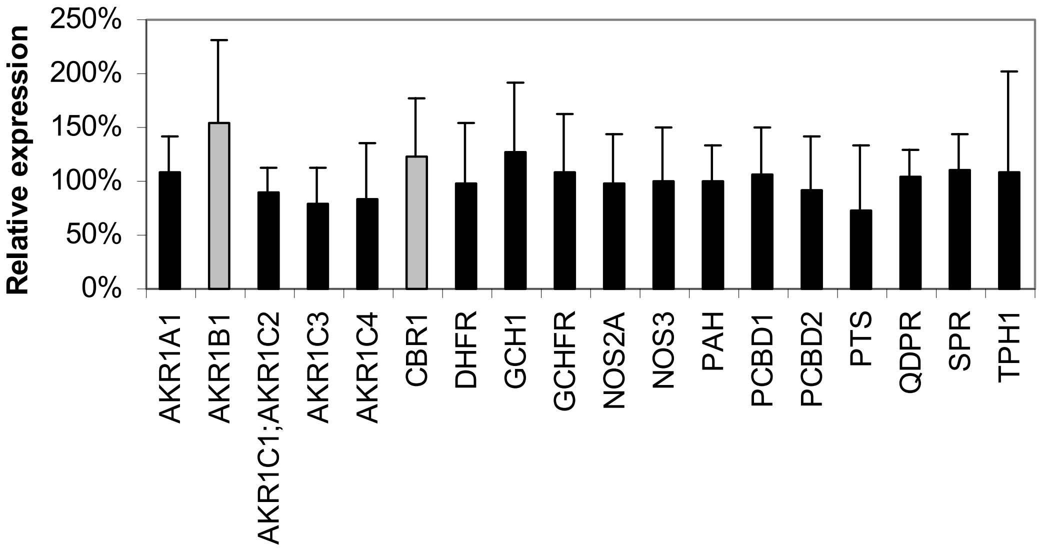

Fig. 12. Gen expression of HepG2 cells: after 24 hours of incubation with cytokines (IFN-γ: 1250 U and TNF-α: 500 U); □ significantly changed expression compared to untreated HepG2 cells (relative expression of untreated HepG2 = 100 %, $p \leq 0.05$); results given as mean activity and standard deviation, (n = 4).

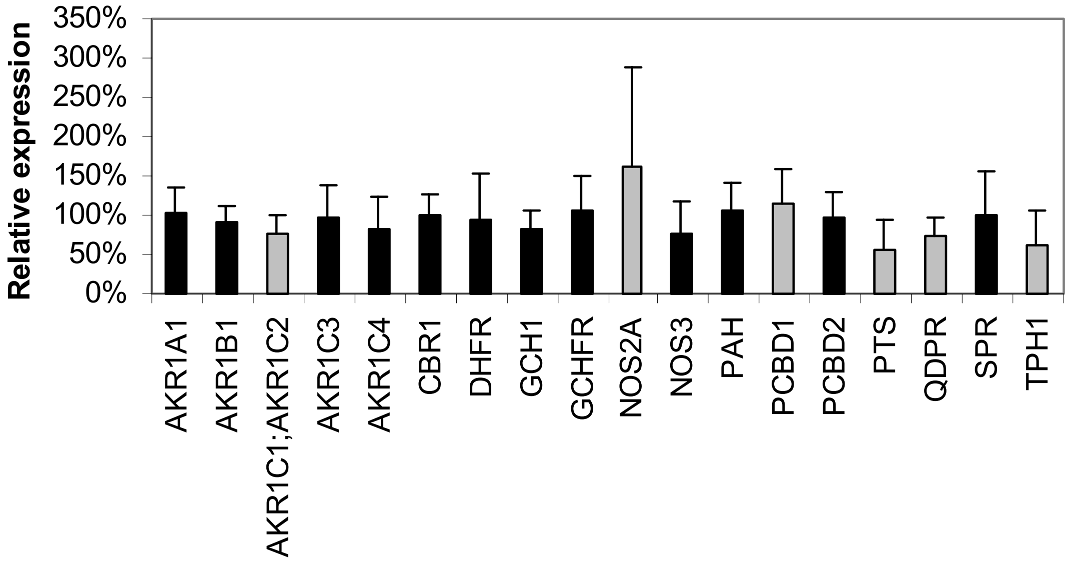

Fig. 13. Gen expression of HepG2 cells: after 24 hours of incubation with 100 µM sepiapterin; □ significantly changed expression compared to untreated HepG2 cells (relative expression of untreated HepG2 = 100 %, $p \leq 0.05$); results given as mean activity and standard deviation, (n = 4).

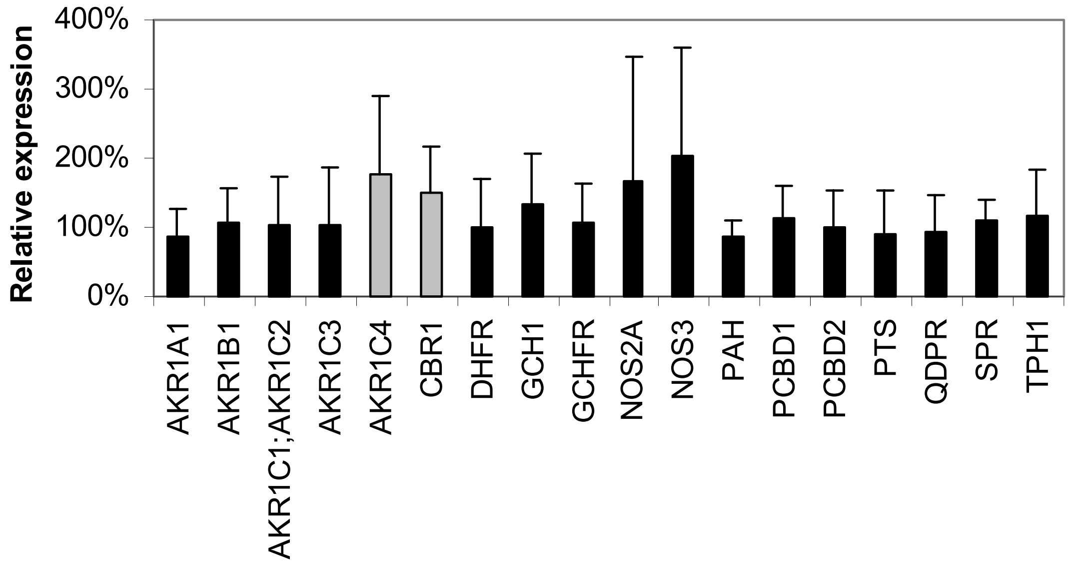

Fig. 14. Gen expression of HepG2 cells: after 6 hours of incubation with 2 mM phenylalanine; ▢ significantly changed expression compared to untreated HepG2 cells (relative expression of untreated HepG2 = 100 %, $p \leq 0.05$); results given as mean activity and standard deviation, (n = 4).

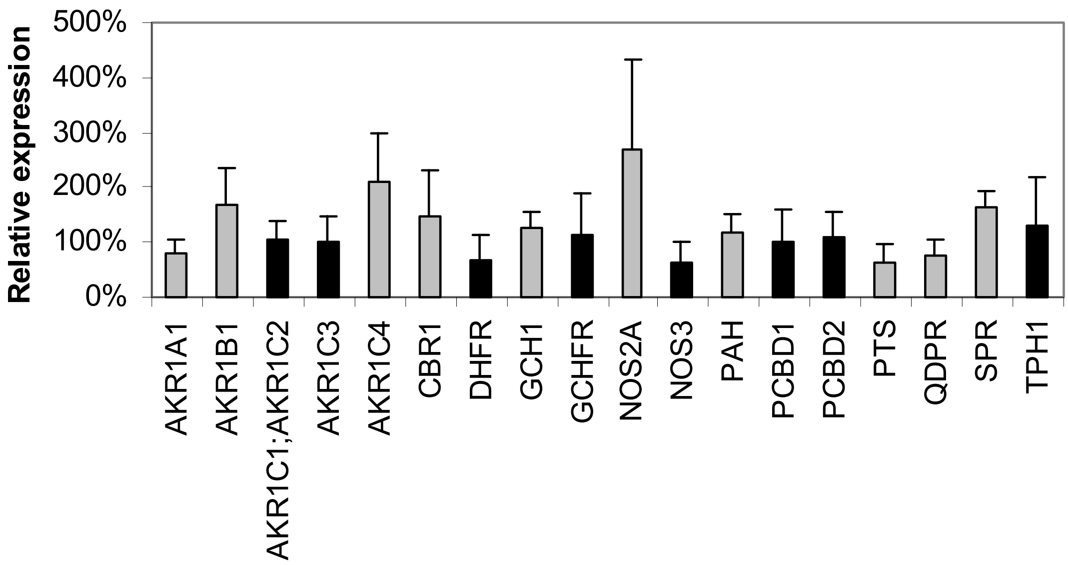

Fig. 15. Gen expression of HepG2 cells: after 24 hours of incubation with 10 mM DAHP, an inhibitor of GTPCH; ▢ significantly changed expression compared to untreated HepG2 cells (relative expression of untreated HepG2 = 100 %, $p \leq 0.05$); results given as mean activity and standard deviation, (n = 4).

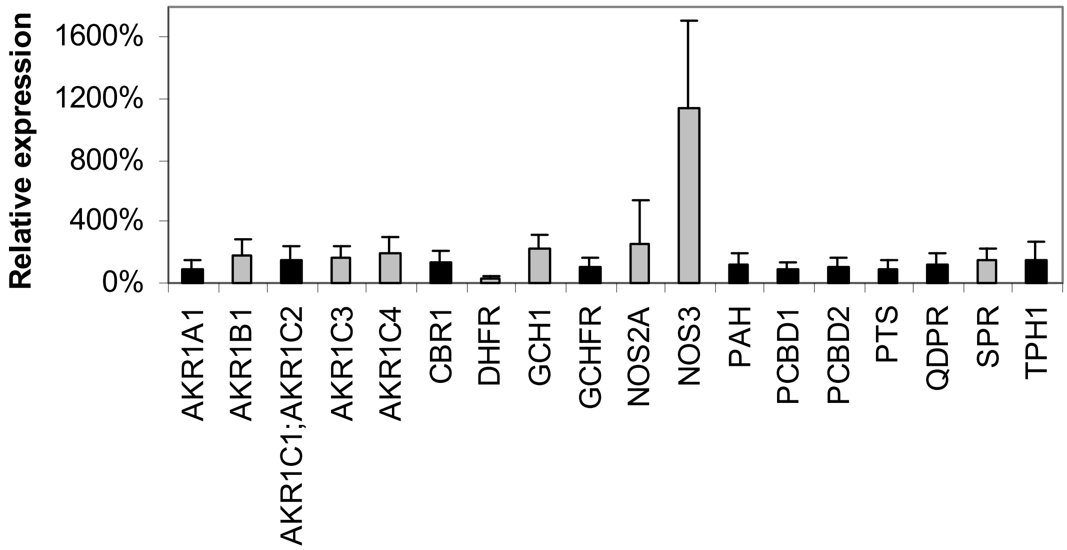

Fig. 16. Gen expression of HepG2 cells: after 24 hours of incubation with 100 µM dicumarol an inhibitor of SR; ☐ significantly changed expression compared to untreated HepG2 cells (relative expression of untreated HepG2 = 100 %, $p \leq 0.05$); results given as mean activity and standard deviation, (n = 4).

We compared gene expression with the enzyme activity of GTPCH and SR and with intracellular biopterin/neopterin production. Most remarkable changes were achieved by treatment of HepG2 with sepiapterin which led to significant raise of the metabolites of the BH_4-metabolism (Figure 17). Intracellular neopterin and biopterin concentration were increased by more than 100-fold, as well was the concentration of these two metabolites in the cell culture medium. But the enzyme activities of the SR and GTPCH were not affected by culturing HepG2 in presence of sepiapterin neither was the relative gene expression of these two enzymes (Figure 17). With exception of dicumarol treatment (inhibitor of SR) the activity of SR was not changed significantly by culturing the cells in presence of cytokines, sepiapterin, phenylalanine or DAHP. Neither was the activity of GTPCH significantly altered by treatment with any of above-named other than GTPCH inhibitor DAHP. No significant effects on the level of enzyme activity were observed using phenylalanine (results not shown).

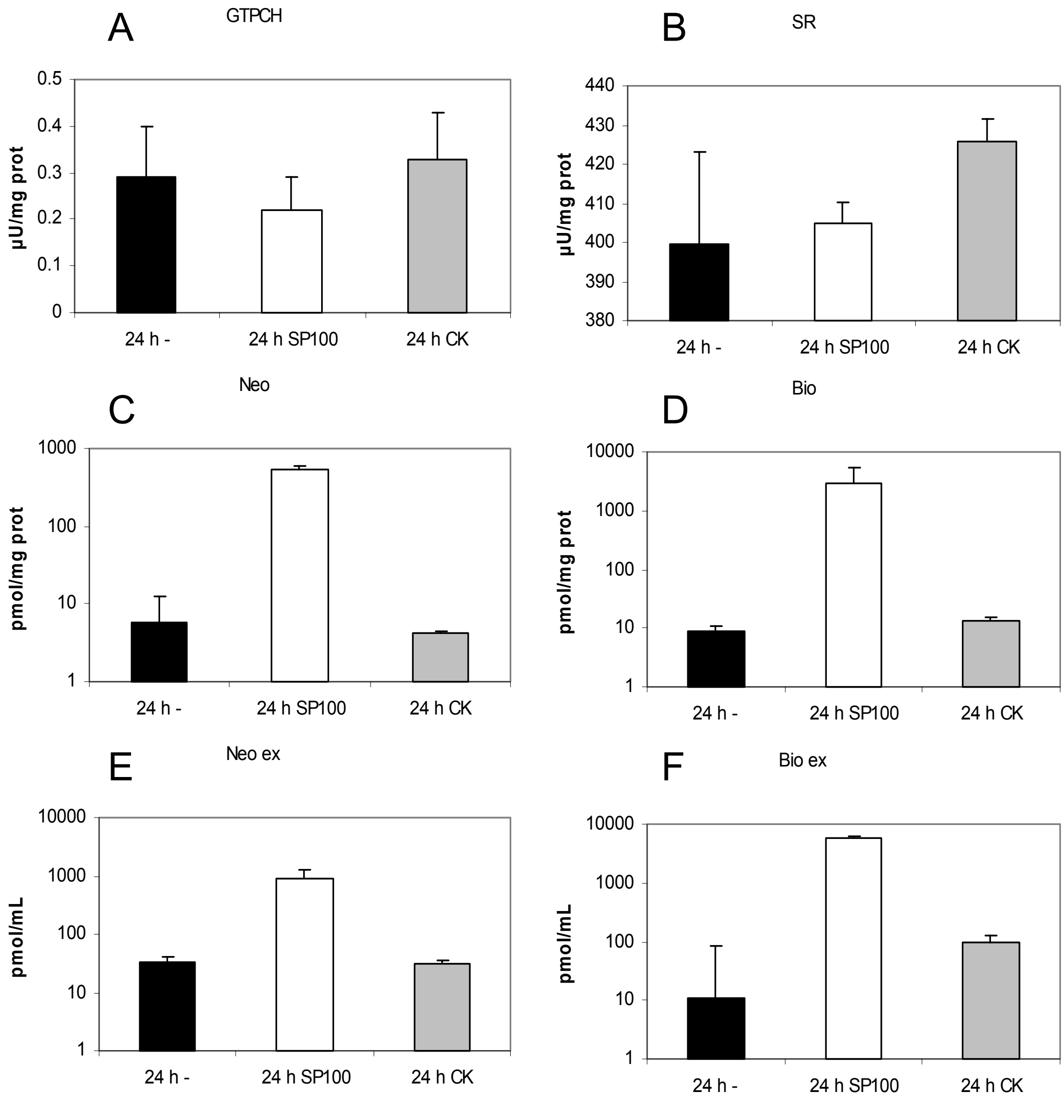

Fig. 17. Enzyme activity assays and neopterin and biopterin measurements in HepG2; results given as mean activity and standard deviation, (n = 3).; ■ untreated controls; □ cells incubated for 24 hours with 100 µM sepiapterin; and □ HepG2 stimulated with TNF-α and IFN-γ. **A)** GTPCH activity; **B)** SR activity; **C)** intracellular neopterin; **D)** intracellular biopterin; **E)** extracellular neopterin; **F)** extracellular biopterin.

Materials and Methods

Following methods and reagents are mentioned in this chapter:

Enzyme assays and measurement of pterins

Following enzyme assays and measurements of metabolites were performed:

GTP cyclohydrolase I (GTPCH) assay

This assay was performed as described in (Hatakeyama and Yoneyama 1998) with some modifications (Bonafé et al. 2001). It measures the conversion of the substrate GTP to neopterin triphosphate by GTPCH; neopterin triphosphate was detected by HPLC after oxidation and dephosphorylation as neopterin.

Cultured cells from confluent 10 cm dishes were trypsinized and centrifuged at 200×g for 4 min, washed by resuspending the cell pellets in 5 mL PBS, then again centrifuged at 200×g for 4 min. The pellets were either kept at −20°C or were quick-frozen in liquid nitrogen and kept at −80°C for longer storage.

The frozen cell pellets were suspended in 200 µL of 1 × homogenization-buffer and lysed by 6 freeze-thaw cycles. Samples were centrifuged at 15000×g (tabletop centrifuge; Biofuge, Heraeus AG) for 5 min at 4°C. Each 150 µL of the recovered supernatant was loaded onto a pre-equilibrated MicroSpin G-25 sephadex column (Amersham Biosciences UK Ltd.) to desalt and remove ATP. They were centrifuged at 735×g for 2 min at 4°C. 50 µL of the filtrate was used in the assay the rest was kept for protein concentration determination according to Bradford method (Bradford 1976). The MicroSpin columns were prepared according to manufacturers instructions, in brief, the resin was resuspended and the bottom closure was snapped off. The column, in a 1.5 mL microfuge tube, was pre-spun for 1 min at 735×g and then equilibrated with 150 µL homogenization-buffer. Shortly before usage, the column was centrifuged again at 735×g for 2 min at 4°C, then loaded with the 150 µL recovered cell lysate and centrifuged as described above.

The enzyme assay was performed as follows; 50 µL of the filtrate were mixed with 148 µL of 1 × reaction buffer and 2 µL of 100 mM GTP in a 1-mL microcentrifuge tube on ice. For duplicates with half amount of protein, 25 µL of filtrate were used. The missing 25 µL were substituted with 25 µL 1 × homogenization buffer. The total volume of 200 µL was divided into two parts of 100 µL. One half was used as test sample the other half was used as blank sample. The test samples were incubated for 1 hour at 37°C in the dark. Additionally a cell-extract-free sample (containing 25 µL of 1 × homogenization buffer plus 1 µL of 100 mM GTP and 74 µL 1 × reaction mixture) was incubated and treated like the test samples. After the incubation, the sample tubes were placed on ice and 10 µL oxidising solution was added. They were incubated for 1 hour at room temperature in the dark. To stop the reaction, 10 µL of ascorbic acid solution were added and mixed with the samples. In contrast to this, blank samples were directly oxidised with 10 µL oxidising solution as above without prior incubation. After 1 hour at room temperature in the dark, oxidation was stopped as well by addition of 10 µL of ascorbic acid. After this step, 14 µL of 1 M NaOH (to adjust the pH to 8.5) plus 20 µL of alkaline phosphatase solution *B* was added to all samples and blanks. This mixture was incubated for 1 hour at 37°C. The reaction was stopped by adding 5 µL of 2 M HCl. The samples (total volume 159 µL) were transferred to an Ultrafree-MC Centrifugal Filter Unit (Millipore Corporation USA, 10000

NMWL) and centrifuged for 15 min at 5000×g at 4°C in a tabletop centrifuge (Biofuge, Heraeus AG). Alternatively samples were centrifuged with YM-10 Microcon Centrifugal Filter Devices (Millipore Corporation USA, 10000 MWCO) at 14000×g for 15 min instead of Ultrafree-MC. The filtrate was stored either at −20°C until analysis or directly analysed for neopterin content using HPLC (liquor-system HPLC, injection volume 10 µL) as described in (Curtius et al. 1991; Zurflüh et al. 2005).

Calculation of the activity: At 37°C one unit of GTPCH produces 1 mol of neopterin per minute. Measured neopterin values [nmol/L] of corresponding blank samples and of the cell-extract-free sample were subtracted from the test samples, then multiplied by 0.106 and divided by the protein concentration [mg/mL] in the desalted lysate. The result was given as µU per mg protein.

Following solution and reagents were used:

- 10 × reaction mixture (diluted to 1 × reaction mixture before use): 50 mL of 1 M Tris-HCl (Fluka), pH 7.5 was mixed with 7.5 g KCl, 2 mL 0.5 M EDTA (Sigma) and 100 mL H_2O.

- 1 × homogenization buffer (freshly prepared before usage): 2 µL 200 mM PMSF (Fluka) were mixed with 1 µL 2 mM leupeptin (Fluka), 1 µL pepstatin (Fluka) and 2 µL 1 M DTT (Fluka) in 2 mL 1 × reaction mixture.

- 200 mM phenylmethanesulphonylfluoride (PMSF; (Fluka)): 34 mg per mL was dissolved in propan-2-ol (Fluka) and stored at room temperature.

- 2 mM leupeptin (Fluka): 1 mg per mL was dissolved in ddH_2O and stored at −20°C.

- 2 mM pepstatin (Fluka): 1.4 mg per mL was dissolved in methanol (Fluka) and stored at −20°C.

- 1 M dithiothreitol (DTT (Fluka)): 15.4 mg was dissolved per 100 µL ddH_2O and stored at −20°C.

- 100 mM GTP Li- salt (Roche): aliquots of 10 µL were stored at −20°C.

- Oxidizing solution: (0.5% iodine / 1% potassium iodide in 1 M HCl): 0.5 g potassium iodide (KI; Fluka) was mixed with 600 µL 1 M HCl and 0.25 g iodine (I_2; Fluka). This mixture was diluted in 50 mL 1 M HCl.

- 2% ascorbic acid solution (Fluka): 2 mg per 100 µL ascorbic acid were dissolved in ddH_2O. This solution was freshly prepared.

- Alkaline phosphatase solution *B*: 220 µL alkaline phosphatase, calf intestine, grade II (Roche Diagnostics GmbH) was mixed with 10 µL 1 M $MgCl_2$, 10 µL 100 mM $ZnCl_2$ and 760 µL 0.1 M Tris-HCl, pH 8. Alternatively 220 µL alkaline phosphatase, calf intestine, grade I (Roche Diagnostics GmbH) diluted 1:10 in 3.2 M ammonium sulphate, 2 mM MgCl and 0.1 M $ZnCl_2$, pH = 7, was used instead. This solution was freshly prepared before usage.

- 100 mM $ZnCl_2$ (Fluka): 13.6 mg $ZnCl_2$ per mL were dissolved in ddH_2O.

- Tris/$MgCl_2$-solution: 1 M Tris-HCl, pH 9.6, 80 mM $MgCl_2$: 46 mL 1 M Tris-HCl, pH 9.6, were mixed with 4 mL 1 M $MgCl_2$ and stored at room temperature.

6-Pyruvoyltetrahydropterin synthase (PTPS) assay

This assay was performed as described in (Shintaku et al. 1988) with modifications (Bonafé et al. 2001).

Cultured cells from a confluent 10 cm plate were harvested by trypsinization, then centrifuged at 200×g (Rotina 46, Hettich Zentrifugen) for 4 min, resuspended in 5 mL PBS and centrifuged again 200×g for 4 min. Cell pellets were either kept at −20°C or quick-frozen in liquid nitrogen and kept at −80°C for longer storage.

The cell pellets were resuspended in 150 µL lysis buffer. After 6 times freeze-thaw-lysis, samples were centrifuged at 15’000×g for 5 min at 4°C in a tabletop centrifuge. Supernatant, containing the cell extract, was recovered.

Samples were prepared in 1-mL microcentrifuge tubes on ice as follows: 50 µL of the cell extract plus 5.5 µL of 200 mM $MgCl_2$ (10 mM final concentration), 5.5 µL 20 mM NADPH (1 mM final), 5.5 µL 20 mM NADH (1 mM final) and 2 µL SR (3 mU), 4.4 µL 50 mU/µL

DHPR (220 mU final), 13 µL NH_2TP (60 µM final) and 24.1 µL of 0.1 M Tris-HCl, pH 7.4 were mixed.

The remaining cell extract was used for protein concentration determination according to Bradford method (Bradford 1976).

Additionally a cell-extract-free blank was produced by mixing the same reagents as above but the 50 µL cell extract were replaced with 50 µL lysis buffer.

Of this mixture each 50 µL were used for the test samples and 50 µL for the blank samples. Test samples were first incubated for 2 hours at 37°C, and then placed on ice. Blank samples were directly placed on ice. The procedure for the remaining steps of the assay was the same for all samples. 15 µL of 30% TCA was added to the samples, which were then centrifuged at 15'000×g for 5 min. After this step 50 µL of the supernatant were recovered and oxidised with 10 µL of a 1% I_2-solution, then incubated for 1 hour at room temperature in the dark. The reaction was stopped by adding 15 µL 1% ascorbic acid to the mixture.

The 75 µL reaction mixture was diluted 1:4 with 225 µL ddH_2O, incubated for 2 hours at 60°C. Afterwards the samples were deproteinized by centrifugation (at 5000×g for 10 min) through Millipore Ultrafree-MC Centrifugal Filter Unit (10000 NMWL).

Diluted samples were analysed by HPLC (Curtius et al. 1991; Zurflüh et al. 2005) for biopterin content (liquor system, 10 µL injection volume).

Calculation of the enzyme activity: One unit of PTPS produces 1 µmol biopterin per min at 37°C. After subtraction of corresponding sample blank and cell-extract-free blank, measured biopterin [nmol/L] concentration was multiplied by 0.036×4 (1:4 dilution) and divided by the protein concentration [mg/mL] leading to the enzyme activity [µU/mg protein].

Following solution and reagents were used:

- Lysis buffer: 0.1 M Tris-HCl, pH 7.4 was diluted 1:10 with ddH_2O and then mixed with 1 mL per 100 mL Triton® X-100 (Fluka) and stored at room temperature.

- 200 mM $MgCl_2$ (Fluka): 4.07 g $MgCl_2 \cdot 6\ H_2O$ was dissolved ad 100 mL with ddH_2O and stored at room temperature.

- 20 mM NADPH (Roche Diagnostics AG): 16.7 mg NADPH was dissolved per 1 mL 0.1 M Tris-HCl, pH 7.4, freshly prepared and kept on ice.

- 20 mM NADH (Roche Diagnostics AG): 14.2 mg NADH was dissolved per 1 mL 0.1 M Tris-HCl, pH 7.4, freshly prepared and kept on ice.

- 3 mU sepiapterin reductase (SR): purified recombinant rat SR (Rec-rSR-His), approx. 1.5 mU/µL stored at −80°C.

- 220 mU dihydropteridine reductase (DHPR; Sigma): sheep liver DHPR, 50 U/mL, stored at −20°C.

- 500 µM dihydroneopterin triphosphate (NH_2TP): produced by using an immobilised GTPCH column, stored at −80°C.

- 0.1 M Tris-HCl, pH 7.4, Fluka: diluted 1:10 with ddH_2O from 1 M Tris-HCl buffer, pH 7.4.

- 1 M Tris-HCl, pH 7.4 (Fluka): 12.1 g Tris were dissolved in ddH_2O ad 100 mL and adjusted to pH 7.4 with HCl.

- 30% trichloroacetic acid (TCA; Fluka): 3 g TCA was dissolved ad 10 mL in ddH_2O.

- 1% iodine (I_2) solution: 2 g potassium iodide (KI; Fluka) and 1 g of iodine (I_2; Fluka) were dissolved in 100 mL ddH_2O and stored at room temperature in the dark.

- 1% ascorbic acid (Fluka): 10 mg ascorbic acid were diluted per 1 mL ddH_2O. Freshly prepared.

Sepiapterin reductase (SR) assay

This assay was performed as described in (Ferre and Naylor 1988) with modifications (Bonafé et al. 2001), it bases on the measurement of the conversion of sepiapterin to dihydrobiopterin (BH_2) by SR. BH_2 is detected as its oxidation product biopterin.

Cultured cells from a confluent 10 cm plate were harvested (1% trypsin), centrifuged at 200×g (Rotina 46, Hettich Zentrifugen) for 4 min, resuspended in 5 ml PBS and then centrifuged again

(200×g, 4 min). Cell pellets were either stored at −20°C for some days or shock frozen in liquid nitrogen and stored at −80°C.

Pellets were resuspended in 1 mL of lysis buffer and lysed by 6 cycles of freezing and thawing. The cell suspension was centrifuged at 15'000×g for 5 min at 4°C in a tabletop centrifuge. The cell extract (supernatant) was recovered.

The assay was performed as follows: Samples were prepared in 1-mL microfuge tubes on ice; 20 µL of the cell extract were mixed with 30 µL ddH_2O and 50 µL of 2 × reaction buffer. This mix was divided into two portions of 50 µL. One half was used for the test samples, the other half for blank samples. In addition to this, a cell-extract-free blank was prepared, containing 10 µL lysis buffer plus 15 µL ddH_2O and 25 µL reaction buffer. A part of the cell extract was used for protein determination in accordance with the Bradford method (Bradford 1976).

The test samples and the cell-free-blank were incubated at 37°C for 30 min in the dark. Then 10 µL oxidising solution were added. Blank samples were immediately oxidised in this way without prior incubation. The oxidation reaction was stopped after a 30 min-incubation at room temperature in the dark by addition of 10 µL 1% ascorbic acid solution.

All samples were diluted 1:2 with 70 µL ddH_2O, then transferred to a Millipore Ultrafree-MC Centrifugal Filter Unit (10000 NMWL) and centrifuged at 5000×g for 15 min. Alternatively they were centrifuged using Millipore YM-10 Microcon Centrifugal Filter Devices at 14000×g for 15 min instead of Ultrafree-MC.

The filtrate was stored at −20°C until analysis with HPLC (urine-system HPLC, injection volume 20 µL), as performed in (Curtius 1991; Zurflüh et al. 2005).

The enzyme activity was calculated as follows: One unit of SR produces 1 µmol of biopterin per min at 37°C, i.e. measured blank sample and cell-extract-free blank values [µmol/L] were subtracted from measured test sample value and then multiplied with 233×2 (1:2 dilution) and divided by the protein concentration [mg/mL]. This resulted in the relative activity of SR [µU/mg protein].

Following solution and reagents were used:

- Lysis buffer: 20 mL 1 M potassium phosphate buffer, pH 6.4 was mixed with 2.2 g KCl (Fluka), 12.5 g glycerol (Fluka), 1 mL 0.5 M EDTA (Sigma) and 200 mL ddH_2O and stored at room temperature.

- 2 × reaction buffer: 100 µL 1 M potassium phosphate buffer, pH 6.4 was freshly mixed before use with 100 µL 1.25 mM sepiapterin (Schircks Laboratories, Switzerland), 100 µL 250 µM NADPH and 200 µL ddH_2O.

- 1 M potassium phosphate buffer, pH 6.4 was prepared by mixing 1 M KH_2PO_4 (Fluka) with 1 M K_2HPO_4 (Fluka) to pH 6.4.

- 1.25 mM sepiapterin: 3 mg sepiapterin (Schircks Laboratories, Switzerland) was dissolved in 10 mL ddH_2O. Concentration was verified using a photometer (ε_λ at 420 nm = 10400 $M^{-1}cm^{-1}$); aliquots of 100 µL in microfuge tubes aerated with N_2 were stored at −80°C.

- 250 µM NADPH (Roche Diagnostics AG): 2.1 mg NADPH were dissolved per mL 0.1 M potassium phosphate buffer, pH 6.4, freshly prepared and kept on ice.

- Oxidation solution: 0.5 g potassium iodide (KI, Fluka) dissolved in 600 µL 1 M HCl was mixed with 0.25 g iodine (I_2, Fluka) and diluted with 50 ml 1 M HCl. It was stored at room temperature in the dark.

- 1% ascorbic acid (Fluka): 10 mg ascorbic acid were diluted per 1 mL ddH_2O. The ascorbic acid solution was freshly prepared before use.

Dihydropteridine reductase assay

This assay was performed as previously described in (Milstien et al. 1976) with some modifications, see (Bonafé et al. 2001).

Cultured cells from 1 confluent 10 cm plate were trypsinized, then centrifuged at 200×g (Rotina 46; Hettich Zentrifugen) for 4 min, resuspended in 5 mL PBS and centrifuged again 200×g for 4 min. Cell pellets were kept either at −20°C or quick-frozen in liquid nitrogen and stored at −80°C within a few days.

Cell pellets were resuspended in 150 µL lysis buffer and disrupted by freezing and thawing six times. The lysate was centrifuged for 5 min at 15'000×g at 4°C in a table top centrifuge. Supernatant was recovered and used in the assay.

Samples were prepared in 1 mL microfuge tubes as follows: 780 µL 50 mM Tris-HCl, pH 7.2 was mixed with 10 µL 2500 U/mL peroxidase (25 U final), 10 µL 880 mM H_2O_2 (8.8 mM final), 100 µL 10mM NADH (1 mM final) and 100 µL of the cell lysate. The remaining lysate was used for the measurement of the protein concentration according to (Bradford 1976).

For each test sample a sample blank was prepared by mixing 880 µL 50 mM Tris-HCl, pH 7.2 with 10 µL 2500 U/mL peroxidase, 10 µL 880 mM H_2O_2 (8.8 mM final) and 100 µL 10 mM NADH (1 mM final). Positive control contained 5 mU sheep liver DHPR (96 U/mL, Sigma) in 880 µL 50 mM Tris-HCl, pH 7.2, 10 µL 2500 U/mL peroxidase, 10 µL 880 mM H_2O_2 (8.8 mM final) and 100 µL 10 mM NADH (1 mM final).

After a 5 min pre-incubation at 25°C the absorbance of NADH at 340 nm of the test and blank samples was measured. Subsequently, 15 µL of 1 mM 6.7-dimethyltetrahydropterin was added as substrate for the DHPR. The samples were quickly mixed and the absorbance against corresponding blanks was measured at 25°C and 340 nm for 5 min. The change in absorption reflexes the consumption of NADH due to the reduction of the substrate 6.7-dimethyltetrahydropterin catalysed by DHPR.

Calculation of the enzyme activity: One unit of DHPR oxidises 1 µmol of NADH per minute at 25°C. The difference of the absorbance was multiplied by 331 and divided by the protein concentration [mg/mL] leading to the relative enzyme activity [mU/mg protein].

Following solution and reagents were used:

- Lysis buffer: 1.49 g KCl was dissolved in 20 mL 50 mM Tris-HCl, pH 7.4, diluted with ddH_2O ad 200 mL and stored at 4°C (all Fluka).
- 880 mM H_2O_2 (Fluka): 5 µL 35% H_2O_2 was mixed with 55 µL 50 mM Tris-HCl, pH 7.2.
- 10 mM NADH: 7.09 mg NADH (Roche Diagnostics AG) was dissolved per mL 50 mM Tris-HCl, pH 7.2. The solution was freshly prepared and kept on ice.

- 5 mU DHPR: 2 µL 96 U/mL sheep liver DHPR (Sigma) were diluted in 998 µL 50 mM Tris-HCl, pH 7.2. Of this dilution 26 µL were mixed with 854 µL 50 mM Tris-HCl, pH 7.2 to get a 5 mU/880 µL DHPR solution.

Phenylalanine hydroxylase (PAH) assay

This assay was adapted from the radiometric PAH assay (Ledley et al. 1987)

Cultured cells from a confluent 10 cm plate were trypsinized, then centrifuged at 200×g (Rotina 46, Hettich Zentrifugen) for 4 min, resuspended in 5 mL PBS and centrifuged again 200×g for 4 min. Cell pellets were either kept at −20°C or quick-frozen in liquid nitrogen and kept at −80°C for longer storage.

Cell pellets were resuspended in 200 µL lysis buffer (adapted from (Christensen et al. 2000)). After 6 times freeze-thaw-lysis, samples were centrifuged at 15'000×g for 5 min at 4°C in a tabletop centrifuge. Supernatants were recovered.

Protein concentration in the supernatants was determined according to Bradford method (Bradford 1976). For the assay generally 50-100 µg of protein was used.

Samples were prepared in microfuge tubes as follows: Blank samples; cell lysates (50-100 µg) were diluted with H_2O to a final volume of 104 µL. They were incubated for 5 min at 95°C in a heating block.

Test samples: The same amount of cell lysate as in the blank samples was diluted with H_2O to a final volume of 77.5 µL. 1 µL 60 mM phenylalanine, 1.5 µL catalase (3.6 U final), 10 µL 2 M potassium-phosphate buffer and 10 µL 1.5 M potassium chloride were added. This mixture was pre-incubated at room temperature for 5 min, then 2 µL 0.1 M dithiothreitol plus 2 µL 4.5 mM 6-MePH_4 was added, followed by a 1-hour incubation at 25°C. The reaction was stopped by placing the samples for 5 min in a 96°C heating block. Thereafter they were put on ice for 5 min.

Test samples and blank samples were spun down at 10'000×g in a tabletop centrifuge. Supernatants were transferred into a Millipore Ultrafree-MC Centrifugal Filter Unit (10000 NMWL) and centrifuged at 5000×g for 10 min.

Phenylalanine and tyrosine levels in the filtrate were measured using an amino acid analyzer (Biochrom 20 Plus; Amersham Pharmacia-Biotech). The PAH activity was calculated as

($\Delta_{[tyrosine]}$ (test sample − blank sample) [µmol/L] × 104 divided by (µL lysate / assay × incubation time [min] × mg/mL protein) = mU PAH / mg protein.

Following solution and reagents were used:

- Lysis buffer: 200 uL 2 M potassium phosphate buffer was mixed with 200 µL 1.5 M KCl, 2 µL 200 mM PMSF, 1 µL 2 M leupeptin, 1 µL 2 M pepstatin and filled up to 2 mL with ddH_2O. The buffer was freshly prepared and kept on ice (all Fluka)

- Homogenisation buffer: 1.15 g potassium chloride (KCl, Fluka) was dissolved in 100 mL ddH_2O, mixed with 5 µL β-SH-EtoH (Fluka) and stored at room temperature.

- 60 mM L-phenylalanine (Fluka): 50 g L-phe was dissolved in 5 mL ddH_2O.

- 4000 U/mL catalase (Sigma): C-100 bovine liver catalase (approx. 50'000 U/mg) was diluted to 4000 U/mL in 10 mM Tris, pH 7.4 and aliquoted into single use tubes. Storage at −20°C.

- 2 M potassium phosphate buffer, pH 6.8: 13.6 g KH_2PO_4 (Fluka) and 22.8 g K_2HPO_4 (Fluka) were dissolved in 100 mL ddH_2O. The buffer was stored at room temperature.

- 1.5 M potassium chloride: 11.18 g KCl (Fluka) was dissolved in 100 mL H_2O.

- 4.5 mM 6-methyltetrahydropterin (Schircks Laboratories): 11.4 mg 6-$MePH_4$ was dissolved in 10 mL degased H_2O. Single use aliquots were stored at −20°C.

- 0.1 M dithiothreitol (Fluka): 15.4 mg DTT was solved in 1 mL ddH_2O and stored in aliquots at −20°C.

- 200 mM phenylmethanesulphonylfluoride (PMSF; Fluka): 34 mg per mL was dissolved in propan-2-ol, Fluka, and stored at room temperature.

- 2 mM leupeptin (Fluka): 1 mg per mL was dissolved in ddH_2O and stored at −20°C.

- 2 mM pepstatin (Fluka): 1.4 mg per mL was dissolved in methanol (Fluka) and stored at −20°C.

Neopterin and biopterin measurement of cell extracts and cell culture medium

Cell extracts were prepared as follows: Cells from 1 confluent 10 cm plate of were trypsinized and centrifuged at 200×g for 4 min then washed by resuspending the cell pellet with 5 mL PBS and again centrifuged at 200×g for 4 min. The pellet was either kept at −20°C or was quick frozen in liquid nitrogen and kept at −80°C for longer storage until use.

The frozen cell pellets were suspended in 200 µL of TED-buffer and lysed by 6 freeze-thaw cycles. Samples were centrifuged at 15000×g (tabletop centrifuge) for 5 min. at 4°C and the supernatants were recovered. 100 µL of the lysate were used in the assay the rest was kept for protein concentration determination according to Bradford method (Bradford 1976).

Samples were prepared as follows: In a 1 mL microcentrifuge tube 100 µL lysate were mixed with 6 µL 1 M HCl and 20 µL 1% I_2 solution and incubated at room temperature for 15 min in the dark (alternatively only 50 µL of lysate plus 50 µL of additional TED-buffer for duplicates with half amount of protein or 50 µL of supernatant cell culture medium plus 50 µL TED-buffer for the measurement of excreted pterins were used). After incubation 20 µL of 1% ascorbic acid solution and 12 µL 1 M Tris-HCl (pH 9.6), 80 mM $MgCl_2$ were added. Following a 1 hour incubation at 37°C, 7 µL 2 M HCl were added and the mixture was transferred to a Millipore Ultrafree-MC Centrifugal Filter Unit (10000 NMWL) and centrifuged for 15 min at 5000×g at 4°C in a tabletop centrifuge. Alternatively samples were centrifuged with Millipore YM-10 Microcon Centrifugal Filter Devices at 14000×g for 15 min instead of Ultrafree-MC. The filtrate was stored either at −20°C until analysis or directly analysed for pterins with the HPLC, urine-system HPLC, injection volume 20 µL as described in (Curtius et al. 1991; Zurflüh et al. 2005).

Calculations: Measured neopterin and biopterin values (in µmol/L) were multiplied by 1750 and divided by the protein concentration in the lysate (in mg/mL). The result was given as pmol per mg protein. In the case of measurement of pterins in the cell culture medium the HPLC result (in µmol/L) was multiplied by 3500 and given as pmol per mL.

Following solution and reagents were used:

- TED-buffer (50 mM Tris-HCl, pH 7.4; 1 mM EDTA; 1 mM DTE): 0.5 mL 1 M Tris-HCl, pH 7.4; 20 µL 0.5 M EDTA (Sigma) plus 1.5 mg DTE (Fluka) were dissolved in 10 mL ddH_2O. 1 mL aliquots were stored at −20°C.

- 1% iodine: 2 g potassium iodide (KI; Fluka) and 1 g of iodine (I_2; Fluka) were dissolved in 100 mL ddH_2O and stored at room temperature in the dark.

- 1% ascorbic acid: 1 mg L-ascorbic acid (Fluka) per 100 µL H_2O was prepared freshly before use.

- 1 M Tris-HCl, pH 9.6, 80 mM $MgCl_2$: 46 mL 1 M Tris-HCl, pH 9.6, were mixed with 4 mL 1 M $MgCl_2$ and stored at room temperature (all Fluka).

- Alkaline phosphatase solution *B*: 220 µL alkaline phosphatase, calf intestine, grade II (Roche Diagnostics GmbH) were mixed with 10 µL 1 M $MgCl_2$, 10 µL 100 mM $ZnCl_2$ and 760 µL 0.1 M Tris-HCl, pH 8. Alternatively 220 µL alkaline phosphatase, calf intestine, grade I (Roche Diagnostics GmbH) diluted 1:10 in 3.2 M ammonium sulphate, 2 mM MgCl and 0.1 M $ZnCl_2$, pH = 7, was used instead. This solution was freshly prepared before usage.

- 2 M HCl (Fluka): 16.7 mL 37% HCl was mixed with 100 mL ddH_2O and stored at room temperature.

- DTE: 1,4-Dithioerythritol (Fluka)

Taurocholate uptake in sandwich-cultured hepatocytes

The uptake assay of bile acid (taurocholate) into hepatocytes was performed as described in (Liu et al. 1998; McRae et al. 2006) with modifications.

Adherent hepatocytes on culture plates were washed 3 times with warm choline buffer (3 mL each, 37°C), and then the cells were incubated in 2 mL taurocholate/choline solution (37°C) for 10, 20, and 30 min at 37°C. For the blank value (0 min incubation) 2 mL cooled (4°C) taurocholate/choline solution was added and aspired immediately after.

The reaction was stopped by 3 times washing the cells with ice-cold choline buffer. Cells were lysed with 2 mL/plate 1% Triton X-100. After a 10 min incubation, cells were detached with a rubber policeman and 1 mL lysate was transferred into a counter vial (the remaining lysate was used for determination of the protein content according to (Bradford 1976)). 5 mL Ultima Gold cocktail (PerkinElmer) were added and the cell lysates were analysed by liquid scintillation spectroscopy.

In parallel the same assay was performed replacing the choline buffer in all steps with a sodium buffer.

As a negative control for the uptake choline buffer containing 1 µM saquinavir with or without 100 µM estrone-3-sulfate were used.

Following solution and reagents were used:

- Sodium buffer: 400 mg KCl, 150 mg KH_2PO_4, 200 mg $MgSO_4 \times 7\ H_2O$, 265 mg $CaCl_2 \times 2\ H_2O$ (Merck), 2.0 g D-glucose (Merck), 4.76 g hepes and 6.8 g NaCl were solved in H_2O to 1 L. pH was adjusted to 7.4.

- Choline buffer: 400 mg KCl, 150 mg KH_2PO_4, 200 mg $MgSO_4 \times 7\ H_2O$, 265 mg $CaCl_2 \times 2\ H_2O$, 2.0 g D-glucose, 4.76 g hepes and 16.20 g choline chloride were solved in H_2O to 1 L. pH was adjusted to 7.4.

- Taurocholate/choline solution: 2.8 µL [^{3}H]taurocholate stock solution (1 mCi/mL) was mixed with 7 µL cold taurocholate stock solution (10 mM) and diluted with 14 mL choline buffer (0.2 Ci/mL and 5 µM final).

- Taurocholate/sodium solution: 2.8 µL [^{3}H]taurocholate stock solution (1 mCi/mL) was mixed with 7 µL cold taurocholate stock solution (10 mM) and diluted with 14 mL sodium buffer (0.2 Ci/mL and 5 µM final).

Cell culture and treatment with different reagents

Cell lines

Following cell lines were employed:

- CCF-STTG 1; homo sapiens brain-, astrocytoma cells (ATCC)

- HCN-1A; homo sapiens brain-, cortical neuron cells (ATCC)

- HepG2; homo sapiens, liver hepatocellular carcinoma (kind gift from Gerd A. Kullak-Ublick Klinische Pharmakologie und Toxikologie)

- HHL-17/HHI-16; Homo sapiens; primary hepatocytes, immortalised with Moloney's mouse leukaemia virus (kind gift from Dr. Arvind Patel MRC Virology Unit, Institute of Virology Glasgow).

- KB; homo sapiens, HeLa contaminant

- SK-N-BE; homo sapiens; bone marrow, neuroblastoma (ATCC)

- THLE-3; Homo sapiens; liver; left lobe; epithelial; immortalised with SV40 large T antigen (ATCC); derived from normal liver cells.

- Fibroblasts; homo sapiens, connective tissue:
  FB Ko 1; normal control fibroblasts
  FB Ko 2; normal control fibroblasts
  FB SRdef, SR deficiency
  FB GCHdef 1, GTPCH deficiency
  FB GCHdef 2, GTPCH deficiency

Thawing of cells

Cells were quickly thawed in a 37°C water bath. Shortly before all ice was gone, cells were suspended usually in 10 mL fresh DMEM medium in a 10 cm plate.

Freezing cells

Adherent cells were washed once with 5 mL PBS (AMIMED, Bioconcept Switzerland) on the plate, then detached with 1 mL 1 × trypsin and incubated for some min at 37°C. The reaction was stopped by adding 5 mL medium. Cell suspension was transferred to 15 mL centrifugation tubes (Techno Plastic Products AG, Switzerland) and spun down at 200×g for 5 min. Supernatant was aspired and the pellet was resuspended in 3 mL ice-cold freezing medium. Aliquots of 1 mL in cryo-tubes, insulated with cotton wool, were kept at −80°C for 3 days and afterwards stored in liquid nitrogen.

Passaging

Cells were usually splitted around 90% confluency. First the cells were washed once with 3 to 5 mL PBS per dish, and then detached with 1 mL trypsin (incubation for approx. 5 min at 37°C).

Cells were splitted into several portions depending on requirements and resuspended each in 10 mL medium per plate.

Cell counting

Confluent cells of one 10 cm plate were trypsinized and suspended in 10 mL medium, then 200 μL of the cell suspension was mixed with 300 μL PBS and 500 μL trypan blue (Bio Whittaker) and incubated for 5 to 15 min at room temperature, then pipetted onto the Neubauer-counting chamber. Cells on 1 big square (1 mm^2 ↔ 0.1 mm^3) were counted and the concentration was calculated: Number of cells per square multiplied by 5 (dilution factor) × 10^4 = Number of cells per mL cell suspension.

DME Medium

One dose of Dulbecco's modified eagle medium (Gibco) plus 18.5 g $NaHCO_3$ (Fluka) were dissolved in 5 L ddH_2O. The pH was adjusted to 7.2 with HCl. The medium was sterile filtered (MediaKap-5 0.2 μm Microgon) which changed the pH to 7.4. Then the DMEM was bottled in 500 mL portions and stored at 4°C. Before use, 50 mL foetal bovine serum (FBS; Gibco) plus 5 mL penicillin/streptomycin was added. Storage at 4°C in the dark.

Mycoplasma staining

Cells were analysed for mycoplasma contamination; cells were cultured on 60 mm plates to approx. 70% confluency. Culture medium was removed and the adherent cells were washed 2 times with 2 mL PBS, then cells were fixed by incubation with 1 mL formaldehyde solution at 37°C for 20 min. The formaldehyde solution was aspirated and cells were permeabilised by washing with cold methanol (−20°C) one time and were then soaked again in 2 mL cold methanol. After an incubation of 30 min at room temperature, the methanol was removed and the culture plate was washed 10 times with H_2O. The cells were dyed with 2 mL staining solution for 15 min at 37°C in the dark, then washed again 10 times with H_2O. Cells were analysed using a fluorescence microscope, 25 × objective immersed in water. Normal cells would show nuclear fluorescence, infected cells a dotted fluorescence in the cytoplasm. Following solution and reagents were used:

Formaldehyde solution: 0.5 mL 37% formaldehyde (Fluka) and 5 μL 1 M $MgCl_2$-solution were diluted with 4.5 mL 1×PBS.

Staining solution: 1 µL of Hoechst stain stock solution was diluted with 10 mL aqueous 0.9% NaCl-solution. The staining solution was freshly prepared before usage.

Reagents used to treat cultured cells

Cultured cells from a ~90% confluent 10 cm dish were incubated for 24 hours with following agents and various mixtures thereof in 5 mL fresh DMEM or other appropriate cell culture medium:

- 2.5 µL IFN-γ (1250 U; Sigma)
- 2.5 µL TNF-α (500 U; Sigma)
- 10-100 µM sepiapterin (Schircks Laboratories) from a 1.25 mM stock solution (stored at –80°C). During handling with sepiapterin, light was dimmed in the working area and cells and sepiapterin were protected from light until the cells were harvested.
- 2-4 mM phenylalanine (Sigma) from a 100 mM stock solution (stored at 4°C) was either incubated for 24 or preferably 6 hours.
- 10 mM 2,4,-diamino-6-hydroxypyrimidine (DAHP; Aldrich) was added from a freshly prepared 20 mM DMEM/DAHP stock solution.
- 100 µM 3.3’-methylene-bis(4-hydroxycoumarin) (dicumarol; Sigma) were added from a freshly prepared 167 µM DMEM/dicumarol stock solution.

Penicillin/streptomycin (penstrep; Gibco): aliquots of 10 mL containing 5000 U penicillin/mL and 5000 µg streptomycin/mL were stored at −20°C.

10 × Trypsin-EDTA (5% trypsin, 5.3 mM EDTA·4 Na; Gibco): 10 × trypsin was diluted 1:10 with ddH_2O, aliquoted in 10 mL and stored at −20°C.

1 × PBS: 10 × PBS (Gibco) was diluted 1:10 with ddH_2O and stored at room temperature.

Freezing Medium (DMEM/10% DMSO/20% FBS): freezing medium was produced by mixing 80 mL DMEM (containing 10% FBS and penstrep) was mixed with 10 mL FBS (Gibco) and 10 mL DMSO (Sigma). Storage at 4°C in the dark.

Interferon-γ human (IFN-γ; Sigma): 1×10^6 U/vial were dissolved in 2 mL PBS (1250 U/2.5 μL). Aliquots of 100 μL were stored at −20°C.

Tumour necrosis factor-α (TNF-α; Sigma): 2×10^5 U/vial were diluted in 1 mL PBS (500 U/2.5 μL) and aliquots of 100 μL were stored at −20°C.

Isolation primary hepatocytes

Liver perfusion was performed using a modified method for a two-step collagenase perfusion (Berry and Friend 1969; Boelsterli et al. 1995; Sidler Pfändler et al. 2004). In short; rats were narcotised with Nembutal (Abbott Laboratories), (0.15 mL/100 g rat). 0.25 mL heparin (500 U/100 g rat) was injected into the femoral vein. The liver was in situ perfused with pre-perfusion solution for 5 min at 37°C. Then the liver was carefully removed and perfused with recycling perfusion solution for 12 min at 37°C. After the liver got soft the liver was placed in a Petri dish with 10 mL ice-cold WME solution A to stop collagenase activity. Thereafter the perfused lobes of the liver were opened and the liver was swivelled in the solution to release the hepatocytes. The cell suspension was filtered twice through a metal sieve (first: 280 μm, 50 mesh; second: 190 μm, 80 mesh) and distributed to 4×50 mL centrifugation tubes. The suspension was filled up to 50 mL with ice-cold WME A medium and centrifuged for 2 min at 4°C and 20×g. Supernatant was aspired, the cells were carefully resuspended with 50 mL ice-cold WME medium A and the centrifugation step was repeated. The supernatant was aspired again and the cell pellet was depending on yield resuspended in 20 mL cold WME medium A.

After isolation of the primary hepatocytes, the number and the vitality of the cells in the suspension were determined using a haemocytometer (Neubauer-counting chamber). 100 μL cell suspension was diluted 1:10 with 0.9% NaCl solution. 450 μL of the dilution were mixed with 50 μL trypan blue (Bio Whittaker) and incubated for exactly 2 min. Thereafter the suspension was pipetted onto the Neubauer-counting chamber. Each 100 cells in three different quadrants were counted and the vitality was given as percentage of unstained (viable) cells to totally counted cells. Then all unstained cells on 4 big squares were counted and the total number of vital cells was calculated (average number of cells per square × 10^6 = number of viable cells in the undiluted suspension/mL).

Cultivation of primary rat hepatocytes

Cells were diluted with WME medium A to a concentration of 0.7×10^6 cells/mL and 3 mL of this suspension was plated per 6 cm collagen coated culture dish (2.1×10^6 cells per dish).

During the first 3 hours the cells were incubated in WME medium A (5% CO_2 (vol/vol), at 37°C) then the medium was changed to WME medium B (3 mL). The WME medium B was thereafter changed daily.

Sandwich cultures using hepatocytes

Hepatocytes sandwich cultures were prepared in a modified version as previously published by Berthiaume et al. and Wang et al. (Berthiaume et al. 1996; Wang et al. 2004). Freshly isolated hepatocytes were plated on 6 cm collagen coated culture dishes as described above. The cells were allowed to attach and 2 hours after the change from WME medium A to B, the medium was aspired, and cells were covered with 150 µL/plate, ice-cold, neutralised collagen solution. After a 1 hour incubation at 37°C to jelly, 3 mL fresh WME medium B was added. WME medium B was renewed daily.

Following solution and reagents were used:

- Hank's balanced salt (HBS) solution: 0.4 g KCl, 0.06 g KH_2PO_4, 8 g NaCl, 0.35 g $NaHCO_3$ (Gibco), 0.048 g $Na_2HPO_4 \times 2\ H_2O$, 1 g D-glucose solved in ddH_2O to 1 L, pH adjusted to 7.4. The solution was sterile filtered and stored at 4°C (all other chemicals; Merck)

- 50 mM Ethylene glycol tetraacetic acid (EGTA; Sigma) stock solution: 19.03 g/L EGTA was solved in ddH_2O; pH was adjusted to 7.4 with 10 mM NaOH. The solution was sterile filtered and stored in 10 mL aliquots at −20°C.

- 1 M HEPES (Sigma): 238.3 g/L HEPES was solved in ddH_2O, pH was adjusted to 7.4 using 10 M NaOH. The solution was sterile filtered and stored in 10 mL aliquots at −20°C.

- Heparin (Sigma) 500 U/100 g rat: Heparin was diluted with 0.9% NaCl to a concentration of 500 U/250 µL 0.9% NaCl. Pre-perfusion solution (400 mL): 4 mL 50 nM EGTA (final 500 µM) was mixed with 8 mL 1 M (final 20 mM), filled up to 400 mL with Hank's (HBS)and sterile filtered.

- Perfusion solution (250 mL) 438.17 mg $NaHCO_3$ was dissolved in 200 mL Hank's (HBS) and mixed with 125 mg BSA (Sigma). 184.42 mg $CaCl_2 \times 2\ H_2O$ was dissolved in 30 mL Hank's (HBS) and slowly mixed with above $NaHCO_3$ solution. The mixture was filled up to 250 mL with Hank's, sterile filtrated and aerated with a 95% O_2/5% CO_2 mixture. 83.3 mg collagenase (25'00 U/250 mL; Type 2 CLS 2; Worthington) was dissolved in 10 mL perfusion solution. Immediately before use, the collagenase was added to the remaining perfusion solution through a 0.45 µm filter.

- Williams medium for hepatocytes (AMIMED, Bioconcept AG): to 500 mL WME, 5 mL L-glutamine (2 mM final; Gibco), 5 mL penicillin/streptomycin (Gibco), 100 U/mL final) and 50 mL FCS (10% final) were added. Storage at 4°C.

- Insulin stock solution (250 U/mL; bovine pancreas; Sigma): 100 mg (24.5 U/mg) was dissolved in 10 mL 0.01 M HCl and stored at 4°C.

- Insulin solution A (25 U/mL): insulin stock solution was diluted 1:10 with 0.01 M HCl – storage at 4°C.

- Insulin solution B (2.5 U/mL): insulin solution A was diluted 1:10 with 0.01 M HCl and kept at 4°C.

- Dexamethasone (0.1 mM; Sigma): a bottle of dexamethasone (1 mg) was dissolved in 277 µL EtOH and diluted with 25.203 mL H_2O. The solution was stored at 4°C.

- WME medium A (seeding medium): 400 mL WME medium (L-Glu/Pen/Strep/FCS) was mixed with 40 µL insulin solution A (2.5 mU/mL final) and 4 mL dexamethasone (1 µM final). The medium was stored and used ice cold.

- WME medium B (culture medium): 500 mL WME medium (L-Glu/Pen/Strep/FCS) was mixed with 50 µL insulin solution B (250 µU/mL final) and 500 µL dexamethasone (0.1 µM final). The medium was stored at 4°C and warmed up to 37°C before use.

- Collagen coated culture plates: collagen R (Serva) parent solution (2 mg/mL) was diluted 1:160 with ddH_2O (12.5 µg/mL final). For 3 cm dishes 1 mL of the above solution was used and for 6 cm plates 2 mL were used. Collagen was poured onto Falcon Primaria plates (BD Biosciences) and incubated for 1 h at 37°C. Afterwards plates were washed 3 times with sterile 0.9% NaCl (2 mL for 3 cm plates and 3 mL for 6 cm, respectively) and stored in a sterile hood overnight. Alternatively pre-coated 6 cm plates from Sarstedt were used.

- Neutralised collagen solution (1 mg/mL): 500 µL collagen R parent solution (2 mg/mL) was diluted with 500 µL 1 M HEPES buffer pH 7.2-7.5 (GIBCO) Ascorbic acid 2-phosphate (Sigma):

Animals

Male Sprague-Dawley rats (RCC) weighing between 210 g and 300 g were used for the isolation of the hepatocytes. The animals had free access to water, were fed ad libitum and housed in a constant alternating 12-hour light (6.30 am to 6.30 pm) and dark cycle. They received human care in accordance with the local animal protection authorities (Sidler Pfändler et al. 2004).

Gene expression experiments

Total RNA isolation from cultured cells

Total RNA was isolated from adherent cultured cells using QIAamp® RNA Blood Mini Kit (QIAGEN) according to supplier's recommendations (QIAGEN 1999). In brief, culture medium was aspirated; cells were washed with 5 mL PBS and then harvested. The cells were either trypsinized or detached using a rubber policeman (only primary rat hepatocytes). 3 mL culture medium was added to the cells, then they were transferred to a 15-mL centrifugation tubes (Sarstedt), and pelleted by centrifugation at 300×g for 5 min. Supernatant was aspirated and the pellet quickly washed with 3 mL PBS (centrifuged again at 300×g for 3 min., supernatant was discarded). Cells were disrupted by mixing with 350-600 µL RLT buffer, containing β-SH-EtOH. The cell lysate was transferred on a QIAshredder spin column and homogenised. 1 volume of 70% ethanol was added to the cell lysate, which was pipetted onto a QIAamp spin column. 350 µL of RW1 buffer was pipetted onto the column and centrifuged. Then, DNA was digested according to appendix D (QIAGEN 2006) in the protocol using 80 µL RNase-free DNase I in RDD buffer (QIAGEN). After a 15-min incubation at room temperature, samples were washed again with 350 µL RW1 buffer. Thereafter 500 µL RPE buffer was pipetted into the column, centrifuged and another 500 µL RPE buffer was added. Samples were centrifuge and to eliminate any chance of possible RPE buffer carryover, columns were transferred into new collection tubes and centrifuged again (optional step 8a in the protocol). Samples were eluted with 30 µL RNase-free H_2O.

Total RNA of the samples quantity was measured in accordance with appendix B (QIAamp® RNA Blood Mini Handbook) carried out with RNA-dilutions in nuclease-free H_2O) and the purity was assessed using RNA-dilution in 10 mM Tris-HCl, pH 7.5, also according to appendix B. A_{260}/A_{280} ratios of 1.9-2.3 were assumed as acceptable.

Total RNA samples were stored at −20°C.

All solutions and reagents were supplied with the QIAamp® RNA Blood Mini Kit.

First-strand cDNA synthesis

cDNA was produced using the Reverse Transcription System (Promega) according to manufacturer's instructions. In brief, samples containing 1 µg total RNA per 9.75 µL nuclease-free H_2O were incubated at 70°C for 10 min in a microfuge tube, then briefly spun down and placed on ice. PCR-tubes containing following mix were prepared: 4 µL 25 mM $MgCl_2$, 10 × reverse transcription buffer, 2 µL 10 mM dNTP mixture, 0.5 µL recombinant RNasin ribonuclease inhibitor, 0.75 µL 20 U/µL AMV reverse Transcriptase (15 U final), 1 µL 500 µg/mL random primers plus 9.75 µL of the before prepared total RNA samples in nuclease free H_2O (50 ng/µL final). The reaction was first incubated for 10 min at room temperature (22°C), then at 42°C for 15 min. After this step, samples were heated to 95°C for 5 min, then cooled down to 4°C for 5 min. All incubation steps were performed on a GeneAmp PCR System 9700 (Applied Biosystems). Synthesised first-strand cDNA was stored at −20°C until use.

All solutions and reagents were supplied with the Reverse Transcription System.

Real time RT-PCR (TaqMan)

TaqMan samples were prepared as follows in 96-well optical plates (MicroAmp Optical 96-Well Reaction Plate; Applied Biosystems): per well 12.5 µL 2 × TaqMan PCR Master Mix (Applied Biosystems) was mixed with 1.25 µL of the appropriate 20 × Assay mix (Biosystems) and 11.25 µL first-strand cDNA in nuclease-free H_2O (25 µL final reaction volume). Usually 30-50 ng cDNA per well were used. All samples were measured in triplicates. The 96-well plate was closed with optical foil (MicroAmp Optical Adhesive Film; Applied Biosystems) briefly spun down at 1500 rpm and then analysed with a ABI Prism 7700 (Applied Biosystems) TaqMan device according to manufacturer's instructions; thermal cycling conditions used were 2 min at 50°C, 10 min at 95°C, (15 s at 95°C, 1 min at 60°C) × 40 cycles. Ct values were exported to MS excel and difference in expression (ΔΔCt-value) of the various genes of the samples were calculated according to the comparative ΔCt-method. Expression was normalised using an endogenous control (either GAPDH or 18S-RNA).

Real-time PCR using Micro Fluidic Cards

RT-PCR was performed according to supplier's guidelines, in brief: first strand cDNA samples were diluted with nuclease-free H_2O to 10 ng/µL. In a 1.5-mL microfuge tube usually 100 ng of first strand cDNA was then diluted with nuclease-free H_2O to a total volume of 50 µL per sample. 50 µL 2×TaqMan Universal PCR Mix (Applied Biosystems) was added and the samples were thoroughly mixed by gentle vortexing. The microfuge tubes were quickly spun down. Then the Micro Fluidic Cards were loaded with the samples; 100 µL of the solution per fill reservoir, eight samples at a time per card.

The Micro Fluidic Card was centrifuged 2 × at 1200 rpm for 1 min (Sorvall Legend T Centrifuge with Sorvall/Heraeus custom buckets and card holder). After centrifugation it was closed using a Micro Fluidic Card Sealer. Filling reservoirs were cut off and the card was run on an ABI Prism 7900HT real-time PCR device (Applied Biosystems). The RT-PCR was performed under following conditions for relative quantification: 2 min at 50°C, 10 min at 94.5°C, then 40 cycles of 30 s at 97°C and 1 min at 59.7°C.

Datasets from the SDS enterprise database were exported as Ct values to MS excel and differences in expression (ΔΔCt-value) of the various genes of the samples were calculated according to the comparative ΔCt-method. Expression was normalised using an endogenous control (either GAPDH, 18S-RNA or ACTB (β-actin)).

Protein analysis

Bradford spectrophotometric method for protein determination

Protein concentrations in cell lysates were determined by Biorad Microassay according to (Bradford 1976) and using bovine γ-globulin as a calibrator.

General buffers, solutions and reagents

2 M HCl (Fluka): 16.7 mL 37% HCl were diluted in 100 mL ddH_2O.

1% ascorbic acid (Fluka): 1 mg L-ascorbic acid per 100 µL H_2O was freshly prepared before use.

Alkaline phosphatase (Roche Diagnostics GmbH), 10 mg/mL containing 1400 U/mL or alkaline phosphatase, calf intestine, grade I (Roche Diagnostics GmbH) diluted 1:10 in 3.2 M ammonium sulphate, 2 mM MgCl and 0.1 M $ZnCl_2$, pH = 7, was used instead. The phosphatase was diluted shortly before usage and stored at 4°C.

2 M HCl: 16.7 mL 37% HCl was mixed with 100 mL ddH_2O and stored at room temperature.

A 10 litre stock of 10x PBS can be prepared by dissolving 800 g NaCl, 20 g KCl, 144 g Na_2HPO_4 and 24 g KH_2PO_4 in 8 L of distilled water, and topping up to 10 L. The pH is ~6.8, but when diluted to 1x PBS it should change to 7.4.

On dilution, the resultant 1x PBS will have a final concentration of 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4

50 mM Tris-HCl, pH 7.2: 6.1 g Tris was dissolved ad 1000 mL with ddH_2O, pH was adjusted to 7.2 using HCl.

Discussion

In the present study, gene expression of proteins and enzymes responsible for BH_4 *de novo* biosynthesis, regeneration and regulation was investigated in different cell systems. Additionally several genes associated with alternative pathways of BH_4 synthesis and of the salvage pathway had been examined. We also engaged in gene expression profile of enzymes which use BH_4 as essential cofactor.

In a first step we attempted to find an cell line which expresses all enzyme activities of the enzymes involved in synthesis and regeneration of BH_4. Most promising were found to be the cell lines SK-N-BE and HepG2, which provided the major activities also in absence of known stimulants.

Many cell lines isolated from tissues of tumour patients present with abnormal metabolism and not all genes of interest may be expressed. Primary rat hepatocytes may serve our purpose and were investigated. Unfortunately, they proved a limited lifetime of GTPCH and PAH activity. We tried to overcome this drawback by culturing these cells in collagen sandwiches. It has been observed that primary hepatocytes, grown between such sandwich layers, expressed over increased periods specific hepatic functions, as the conditions are much closer to the *in vivo* environment compared to normal 2D-monolayers (Berthiaume et al. 1996; Sidler Pfändler et al. 2004; Wang et al. 2002, 2004). In our hands, even though higher than in conventional monolayer controls, GTPCH activity could not be maintained employing such sandwich cultures as the enzyme activity was not stable over 24 hours and rapidly decayed. Also the fact that primary rat hepatocytes had to be cultured in presence of 0.1 µM dexamethasone and 250 µU/mL insulin, which both interfere with BH_4-synthesis, let us turn away from these cells (Ishii et al. 2001; Skimming et al. 2003).

We concentrated on cell lines SK-N-BE and HepG2 but used also additional cell lines of hepatic origin (HHL-17 and HH-16) which were isolated from the liver of a healthy subject. These human primary hepatocytes were immortalised with Moloney's mouse leukaemia virus. Furthermore, we analysed fibroblasts of different BH_4-deficient patients (SR-deficiency and GTPCH-deficiency) and healthy controls. We used a novel format of TaqMan RT-PCR, so-called Micro Fluidic Cards (Applied Biosystems) to estimate gene expression. Amplification was measured in duplicates and single samples or samples of the same cell type and same treatment were measured multiple times (between 2 and 5 times) and resulted in some cases in considerable variations of calculated expression. Particularly genes like *GCHRR*, *PTS*, *AKR1C3* or *DHFR* were sometimes problematic to assess, being expressed either at the border of detection limit or giving inconsistent results. Strong expression was found for *GCH1* (in the average about 50-fold induction), *AKR1B1* (average induction about 3 to 4-fold compared to untreated controls) both induced by cytokines and a milder down-regulation of the expression, to 80% of levels in untreated controls, of the gene *QDPR*. Not only on the level of gene expression but also in regard of the enzyme assays and metabolite measurements were considerable variation observed.

A closer look at the expression pattern of fibroblasts of patients and controls, with and without supplementation of cytokines revealed at least in patients with GTPCH deficiency a significant reduction of *GCHFR* expression. Whether the same holds also true for normal controls or on the level of protein production could not yet be verified. A similar observation was made upon stimulation cell cultures with lipopolysaccharide (LPS) by Werner et al. (Werner et al. 2002). They found a down-regulation of *GCHFR* expression in cultured human cells as well as in rats treated with LPS *in vivo.* They had anticipated that *GCHFR* expression would be induced together with the protein it regulates, *i.e.* with GTPCH. The opposite effect was found, rendering GTPCH in the cells independent of the metabolic control by phenylalanine. One possible explanation is that in a situation where expression of GTPCH is triggered as a response of immunological signals (like proinflammatory cytokines or LPS) metabolic regulation, as accomplished by phenylalanine in the interplay with GFRP, is not necessary and can be circumvented by repression of *GCHFR*, the gene coding for GFRP.

In a last series of experiments, we focused on HepG2 cells which were treated with cytokines, sepiapterin, phenylalanine, DAHP (inhibitor GTPCH via interaction with GFRP), dicumarol (inhibitor of SR) and virtually all possible combinations of those reagents. Gene expression was assessed using Micro Fluidic Cards and the cells were analysed for metabolites and enzyme activity of GTPCH and GFRP. As seen in the experiments above, considerable variation in gene expression of samples of the same type were observed. *AKR1B1* responded to most agents with induction of gene expression and the function of activation of mRNA production remains unclear. In Parkinson's patients an inverse reciprocal expression pattern of *SPR* and *AKR1B1* was observed (Tobin et al. 2007); such a correlation between the two could not be found in our study. Further, this gene was associated with potential control of stress response in experiments with *Saccharomyces cerevisiae* (Chang et al. 2003), being a general regulator of stress response might also serve as a potential explanation in our case. As *GCH1* is constitutively active in HepG2 cell, the change in expression of the gene was markedly lower compared to the change in fibroblasts. Similarly as found in the Hep3B hepatoma cell line, biopterin and neopterin concentration drop in our cells closely to zero after treatment with DAHP or with dicumarol but in contrast to Aguado and others (Aguado 2006) we did not observe reduced gene expression of the *PAH* gene. There are additional observations concerning gene expression in HepG2 cell which need further investigation.

Chapter **7**

DISCUSSION

Outcome and Outlook

In the here presented Ph.D. thesis, we engaged through a series of studies, metabolism, regulation, pharmacokinetic characteristics and implications of BH_4 for patients with either BH_4-responsive PKU/HPA or a BH_4-deficiency. Chapters 2 to 6 are discussed in more detail at the end of the corresponding section. Here a brief summary.

In chapter 2, *Screening for tetrahydrobiopterin deficiencies using dried blood spots on filter paper*, we described a novel method for the analysis of pterins. Dried blood spots on filter paper (Guthrie cards) were introduced in the early 1960s for newborn screening, including the metabolic disease PKU. With the introduction of tandem mass spectrometry, a number of new tests were developed for blood spots. Analysis using dried blood spots became a useful alternative to measurements of serum, plasma or urine samples. Only minimal sample volumes are needed and specimens can be sent in an envelope by ordinary mail. Blood spots are used to measure DHPR activity in patients with HPA for the selective screening of BH_4 deficiencies. Up to date analysis of BH_4 metabolites is performed using urine. According to the profile of neo- and biopterin in urine, enzyme defects of BH_4 metabolism can be localised. One major problem of analysis of pterins in body fluids is their sensitivity to light and oxygen; samples need to be sent frozen on dry ice or fully oxidised to the laboratory performing the analysis. In dried blood spots on filter paper, BH_4 is already fully oxidised to biopterin and partially degraded to pterin. Once oxidised, pterins are stable in blood spots for about up to two weeks at room temperature. It was estimated that this time should be sufficient to send a sample to the laboratory.

The method we developed, allows measuring amino acids, DHPR activity and pterins from a single Guthrie card.

Elution and extraction of pterins was optimised and recovery was calculated to be 63-69%. Concentrations of pterins from blood spots were expressed per haemoglobin, as a relatively high amount of neopterin and biopterin is located in erythrocytes.

Blood spots biopterin + pterin were used to follow BH_4 pharmacokinetics in healthy controls and patients with HPA. In a pilot study of more than 70 patients with HPA, we measured blood spot pterins before loading test and compared results with the standard screening method using urine. The profile of pterins in blood spots was identical with that found in urine collected at the same time. Our preliminary results suggested that blood spots on filter paper may be a practical alternative in differential screening of BH_4 deficiencies.

In chapter 3, *Pharmacokinetics of orally administered tetrahydrobiopterin in patients with phenylalanine hydroxylase deficiency*, we studied pharmacokinetic parameters of BH_4 in PAH-deficient patients. Even though extensive pharmacokinetic studies have been performed in animals, not much was known about the situation in humans. We analysed dried blood spots of patients after BH_4 loading test. Plasma profile of total biopterin showed a first-order kinetic, with a fast absorption phase and a rapid decline in the distribution phase followed by a slower decline in the final elimination time. Similarly to what was reported in healthy controls, blood BH_4 peaked at 4 hours in 90% of the patients.

Data from combined Phe+BH_4 loading test showed that administration of phenylalanine (100 mg/kg) almost doubled blood BH_4 concentrations after 3 hours in about 70% of the patients. This is consistent with previous findings that biopterin concentration in urine or plasma correlates with blood phenylalanine concentrations.

In the chapter 4, *Molecular genetics of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency*, mutations from BH_4-responsive patients (315) collected in the BIOPKU database were analysed for association with BH_4 responsiveness. Alleles and genotypes, defined as BH_4-responsive, were compared with data previously reported for PKU patients in several European countries, Northern China and South Korea. Furthermore, the PAHdb knowledgebase, compiling 3171 mutant alleles from all over the world, was analysed. Comparison of different databases and PKU studies with the BIOPKUdb allowed us to estimate both allele and genotype frequencies of BH_4-responsiveness.

BH_4-responsiveness seems to be multifactorial, a stabilising effect of BH_4 (chaperone-like activity) on PAH, being probably the major mechanism. It was proposed that the responsiveness to BH_4 in patients with PAH deficiency is probably due to suboptimal physiological concentrations of BH_4 in hepatocytes (Kure et al. 2004).

Our findings reveal several features common to different patient groups; both the frequency of potentially BH_4-responsive alleles and genotypes is higher than initially assumed from the loading test studies; the BH_4 responsiveness is characterised by a substantial residual PAH activity of at least one mutant allele.

Several authors reported inconsistency of BH_4-responsiveness within the same genotypes and questioned the genotype-phenotype correlation. Especially genotypes harbouring the mutations p.L48S, p.I65T, p.R158Q, p.R216Q, and p.Y414C lead frequently to controversial results. In addition, it has been reported that some patients who responded in the single loading test did less well on a continuous supplementation with BH_4.

Although there is no absolute genotype-phenotype correlation, mutation analysis provides useful information on potential non-responders in patients harbouring two null alleles and may, to some extent, predict possible BH_4-responders.

The chapter 5, *Outcome and long-term follow-up of 36 patients with tetrahydrobiopterin deficiency*, dealt with treatment, clinical, and biochemical findings and the outcome of 26 patients with PTPS deficiency and 10 patients with DHPR deficiency.

Today selective screening for BH_4 deficiencies is performed in most parts of the world in patients with even slightly increased phenylalanine levels detected in the newborn screening. Exact diagnosis of the type of BH_4 deficiency can be made in the first weeks of life. This led to an early onset of therapy and prevention of the severe mental and physical disability reported of untreated patients. Early diagnosis is associated with better outcome. However treatment guidelines are vague, reports of long-term follow-up and outcome are rare and inconsistent.

In the group of PTPS patients analysed in this study there was no clear correlation between any one factor and a good outcome. Indications were found that Selegiline in some cases allowed a lower dosage of neurotransmitter thus not only the on-off effects and the daily fluctuations diminished or disappeared, but probably also the known side effects of the long-term treatment.

Early diagnosis and correct, closely monitored therapy can reduce symptoms and prevent catastrophic outcome; however it remains uncertain, which factor decides whether a child will experience delayed development and which not.

In the case of DHPR-deficiency an early diagnosis and early introduction of therapy in the first months of life appears to be crucial, as permanent brain damage occurs early in the course of the untreated disease. Analysis of the 10 patients supported this, suggesting a cut-off time limit for the therapeutic intervention of one month of life.

In the last chapter 6, *Gene expression of BH_4 treated cells*, preliminary results from studies assessing the gene expression profile of enzymes involved in the biosynthesis and in the recycling of the cofactor BH_4 and of enzymes depending on it, are described.

In a first step we tried to find a cell line, which expresses all enzymes involved in synthesis and regeneration of BH_4. Most promising were the cell lines SK-N-BE and HepG2 which provided activities of all BH_4 enzymes.

Trying to reduce artificial effects on the level of the metabolism and gene expression, we investigated also in the use of primary cells, i.e. primary rat hepatocytes. As they proved a limited lifetime of PAH and GTPCH activity, culturing these cells in collagen sandwiches, which had been observed to reflect a condition much closer to the *in vivo* environment compared to normal 2D-monolayers, was tried. The GTPCH activity was slightly higher than in conventional monolayer controls, but could not be maintained, employing such sandwich cultures, for over 24 hour.

In addition to SK-N-BE and HepG2 we used cell lines of hepatic origin (HHL-17 and HH-16) which were isolated from the liver of a healthy subject. Furthermore, we analysed fibroblasts of different BH_4-deficient patients. We applied a novel format of TaqMan RT-PCR, so-called Micro Fluidic Cards (Applied Biosystems) to estimate gene expression. A marked change of expression was found for *GCH1* and *AKR1B1* both induced by cytokines and a milder gene expression down-regulation, to 80% of levels in untreated controls, of the gene *QDPR* by supplementation with sepiapterin. Not only on the level of gene expression but also in regard of the enzyme assays and metabolite measurements were considerable variation observed.

A closer look at the expression pattern of fibroblasts of patients and controls, with and without supplementation of cytokines revealed at least in patients with GTPCH deficiency a significant reduction ($p \leq 0.05$) of *GCHFR* expression. Whether the same holds also true for normal controls or on the level of protein production could not yet be verified. A similar observation was made upon stimulation cell cultures with lipopolysaccharide (LPS). A down-regulation of *GCHFR* expression in cultured human cells was found as well as in rats treated with LPS *in vivo* (Werner et al. 2002).

In a last series, we focused on HepG2 cells which were treated with cytokines, sepiapterin, phenylalanine, DAHP (inhibitor GTPCH via interaction with GFRP), dicumarol (inhibitor of SR) and virtually all possible combinations of those reagents. Gene expression was assessed using Micro Fluidic Cards and the cells were analysed for metabolites and enzyme activity of GTPCH and SR. As seen in the experiments before, considerable variation in gene expression of samples of the same type were observed. *AKR1B1* responded to most agents with induction of gene expression and the function of activation of mRNA production remains unclear. This gene was associated with potential control of stress response in experiments with *Saccharomyces cerevisiae* (Chang et al. 2003), being a general regulator of stress response might also serve as a potential explanation in our case. As *GCH1* is constitutively active in HepG2 cell, such a marked change in expression of the gene as in fibroblasts, was not observed. But by supplementation with DAHP and dicumarol the expression was increased, potentially as a response to increased inhibition of the enzyme through GFRP/DAHP and in the second case as answer to inhibition of sepiapterin reducing BH_4 synthesis. Similarly as found in the Hep3B hepatoma cell line, biopterin and neopterin concentration drop in our cells closely to zero after treatment with DAHP or with dicumarol but in contrast to Aguado and others (Aguado et al. 2006) we did not observe reduced gene expression of the *PAH* gene. There are additional observations concerning gene expression in HepG2 cell which need further investigation.

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SEVERE MUCITIS AFTER SUBLINGUAL ADMINISTRATION OF TETRAHYDROBIOPTERIN IN A PATIENT WITH TETRAHYDROBIO-PTERIN-RESPONSIVE PHENYLKETONURIA

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Tetrahydrobiopterin (BH_4) is the cofactor for phenylalanine hydroxylase (PAH) and is essential to treat different forms of hyperphenylalaninaemia, i.e. patients with defects in the biosynthesis of BH_4, but also in the PAH gene (Bernegger and Blau 2002; Kure et al. 1999; Muntau et al. 2002). Responsiveness of mutant PAH to BH_4 administration relies on a chemical chaperon effect of BH_4 preventing PAH from protein misfolding and inactivation (Thöny et al. 2004), but several other mechanisms may also be involved (Blau and Erlandsen 2004). Recently, we reported that sublingual administration of BH_4 tablets in a single control person resulted in 58%–67% higher plasma BH_4 concentrations than the usual oral route (Fiege et al. 2004). Drawbacks included rapid decomposition of tablets, acidic taste, and increased salivation. Since sublingual application could reduce therapy costs, orange flavoured artificially sweetened BH_4 pastilles were tested on four healthy volunteers and a child with mild phenylketonuria (PKU).

BH_4 (Schircks, Switzerland) was administered to four healthy male volunteers (age 28–57 years) on 2 subsequent days either as tablets (50 mg) or pastilles (100 mg, con

taining 5 mg aspartame and orange aroma) with a 1 week wash-out between the two trials. Blood was collected before, and 1, 2, 3, and 4 h after administration. An 8-year-old patient with mild PKU (genotype P281L/R261Q) who was already on regular BH_4 therapy was treated with BH_4 pastilles (sublingually, 12 mg/kg/day; bid) in addition to P-AM 2 formula for 2 weeks. Written consensus was obtained from the family.

In the four healthy male volunteers, total biopterin values before and up to 4 h after oral and sublingual administration were compared (Fig. 1A). No statistically significant difference between oral and sublingual administration was observed. Pterin concentrations increased after both oral and sublingual administration, reflecting instability of BH_4 in blood. The ratio of pterin to cumulated biopterin and pterin ranged between 22.8% and 33.5%. Summing biopterin and pterin instead of total biopterin did not alter the ratio between oral and sublingual administration (data not shown).

Changing from standard therapy to sublingual administration in a child with mild PKU resulted in almost unchanged plasma phenylalanine concentrations (Fig. 1B). After 2 weeks of twice daily sublingual BH_4 administration, the patient complained of a painful prickling sensation at the tip of the tongue. He nevertheless continued the treatment for a further 10 days, moving the tablets in his mouth from one side to the other, which resulted in severe mucitis. His condition normalised rapidly upon discontinuation of the sublingual form. No mucitis appeared in healthy volunteers.

A clinical trial with sublingual BH_4 in a patient with mild PKU failed despite pharmacological effectiveness on blood phenylalanine levels because of an adverse pastille-related side-effect. Formulation of buffered tablets approaching neutral pH and further investigations on BH_4 metabolism and pharmacokinetics are needed.

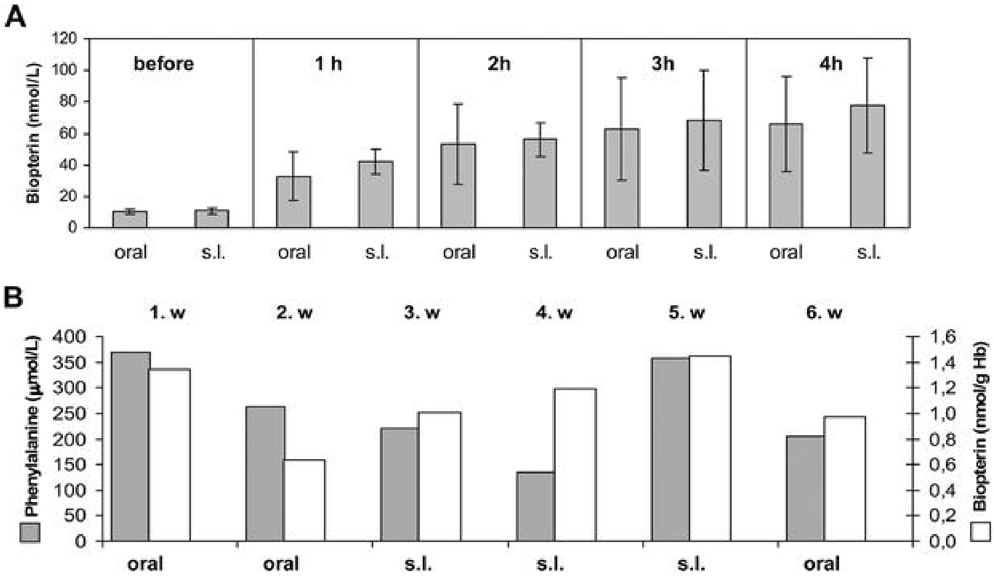

Fig. 1. (A) Comparison of total plasma biopterin levels (median ± SD) after oral or sublingual administration of BH_4 (2 mg/kg) in four healthy volunteers at different time points. (B) Dried blood spot phenylalanine (*solid squares*) and biopterin (*open squares*) measured 3 h after oral (15 mg/kg) or sublingual (12 mg/kg) administration of BH_4 in a patient with mild PKU (*s.l.* sublingual)

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